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Comparison of Mycoplasma hyopneumoniae strains by serologic and electrophoretic methods

Lee-Hwa Lisa Ro
Iowa State University

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by

Lee-Hwa Lisa Ro

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INTRODUCTION

Mycoplasma hyopneumoniae is the etiological agent of mycoplasmal pneumonia of swine or enzootic pneumonia of pigs. The disease is very common all over the world and causes economic losses through stunted growth and poor food efficiency of swine. Surveys in different countries have indicated that 30-80% of slaughter pigs have typical mycoplasmal pneumonia lesions (Whittlestone, 1973, 1979; Switzer and Ross, 1975). Braude and Plonka (1975) estimated that Mycoplasma hyopneumoniae disease caused an average 0.75 pound loss per pig raised in England.

In recent years, selective media and isolation techniques for primary isolation of Mycoplasma hyopneumoniae have been improved (Friis, 1975, 1979; Goodwin, 1976; Etheridge et al., 1979). Also, the identification and classification of mycoplasmas has been increasingly dependent on serological and electrophoretic techniques. These tests have demonstrated the existence of serological and electrophoretic heterogeneity among strains within a mycoplasmal species and of course have demonstrated differences between mycoplasma species. It is especially important to determine the degree of relatedness or distinctiveness when one attempts identification of new isolates.

In this study, Mycoplasma hyopneumoniae strains were

isolated from pneumonic lung of pigs and were cloned 3 times. Six field strains of Mycoplasma hyopneumoniae, strain 11 of Mycoplasma hyopneumoniae, J strain of Mycoplasma hyopneumoniae, Ms 42 strain of Mycoplasma flocculare and BTS 7 strain of Mycoplasma hyorhinis were compared by means of serological tests and electrophoretic techniques in order to determine whether any heterogeneity existed among Mycoplasma hyopneumoniae strains.

LITERATURE REVIEW

History and Taxonomy

In 1931, Shope reported a disease in swine differing from hog flu. The disease was more chronic than swine influenza. Later, Köbe (1933), Lamont (1938), Pullar (1948) and Gulrajani and Beveridge (1951) reported a chronic pneumonia of swine which differed from swine influenza distributed in Germany, Ireland, Australia and England, respectively. Betts (1952) suggested the name "virus pneumonia of pigs" for the disease described by Gulrajani and Beveridge (1951). Hjärre et al. (1952) reported an agent differing from swine influenza virus which caused pneumonia of swine in Sweden, and named the disease "enzootic virus pneumonia of pigs".

Beveridge and Betts (1953) demonstrated that a filterable agent was the causative agent of a chronic pneumonia of swine. Wesslén and Lannek (1954) used tissue culture technique and recovered organisms from catarrhal pneumonia of swine; the organisms were referred to as swine enzootic pneumonia agents. In 1957, Lannek and Wesslén (1957) suggested that the etiological agent of enzootic pneumonia might be a pleuropneumonia-like organism (PPLO). Also, Whittlestone (1957, 1958) found bodies resembling PPLO in touch preparations from fresh pneumonic tissue.

L'Ecuyer (1962), L'Ecuyer and Switzer (1963), Goodwin and Whittlestone (1963) and Betts and Whittlestone (1963) reported the successful propagation of agents they had isolated from swine pneumonia in cell cultures. Subsequently, pneumonia was produced in pigs with an organism grown in cell-free medium by Goodwin and Whittlestone (1964); and the term "enzootic pneumonia of pigs" was adopted by them.

In 1965, Maré and Switzer demonstrated the VPP 11 strain which L'Ecuyer and Switzer (1963) isolated from "virus pneumonia of pigs" as the pulmonary pathogen of swine with cell-free medium, and the organism was named M. hyopneumoniae. A few months later, Goodwin et al. (1965) reported the formation of mycoplasma colonies on solid medium inoculated with their J strain. These organisms induced enzootic pneumonia in pigs and they proposed the name M. suipneumoniae for their isolate. The name "mycoplasmal pneumonia of swine" was suggested for the disease by Maré and Switzer (1966b).

Strain 11 of M. hyopneumoniae and the J strain of M. suipneumoniae were found to be serologically indistinguishable by means of the growth inhibition and metabolic inhibition tests (Goodwin et al., 1967). Hodges et al. (1969) also reported that the two strains were indistinguishable with the metabolic inhibition test.

Two names for the same mycoplasma have been used since 1965. In 1974, the International Committee on Systematic Bacteriology (ICSB) Subcommittee on the Taxonomy of Mycoplasmatales (1975) noted that M. hyopneumoniae had priority, and suggested that the J strain of M. suipneumoniae be the neotype strain for M. hyopneumoniae. Rose et al. (1979) reported that strain 11 of M. hyopneumoniae and the J strain of M. suipneumoniae were serologically and biologically indistinguishable. They supported the recommendation from the ICSB Subcommittee.

More details about the history and taxonomy of M. hyopneumoniae have been reviewed by Maré (1965), Huhn (1969), Amanfu (1980) and Piffer (1981).

Morphology

Light microscopic examination of M. hyopneumoniae grown in liquid media and stained with May-Grunwald-Giemsa stain revealed small ring and bipolar organisms and larger bodies (5.5 μ dia.) with deeply stained internal structures and condensations (L'Ecuyer, 1969; Whittlestone, 1973). Maré and Switzer (1965) reported that films of M. hyopneumoniae contained coccoid (0.3-0.5 μ dia.) and ring forms. The organisms had the tendency to grow in colonies (10-25 μ dia.) on glass coverslips immersed in broth

(Goodwin and Whittlestone, 1966; Goodwin et al., 1967). Colonies consisted mainly of cocci (0.5 μ dia.) strung on fine filaments (0.1-0.2 μ dia.) like branching necklaces. Globular forms (6 μ dia.) also were detected in some colonies. In Giemsa-stained coverslip preparations, L'Ecuyer (1969) found cocci (0.5 μ dia.) in early passages and pleomorphic organisms (0.85-2.0 μ dia.) with unipolar or bipolar dense areas in later passages.

In Giemsa-stained touch preparations of lung, the organisms appeared as delicate rings (0.5 μ dia.) with thickenings at one or more points, and as bipolar organisms (0.5-1.0 μ dia. in length). Cocci (0.2-0.5 μ dia.), triangles (1.0 μ dia.) and pointed rings (2.0 μ dia.) also were seen but were not common (Maré and Switzer, 1966a; Whittlestone, 1973).

In cell cultures, M. hyopneumoniae occurred extracellularly. The organisms were pleomorphic forms containing cocci (0.5 μ dia.) and rings (0.6-3 μ dia.). The cocci were strung on fine branching filaments (0.1 μ dia.). The organisms were usually detected in lung cell cultures on the 2nd or 3rd day after inoculation (Goodwin and Whittlestone, 1963; Whittlestone, 1973; Pijoan, 1975).

On solid media, the organisms grew in a 5-10% CO_2 atmosphere but not in aerobic conditions (Goodwin et al., 1965). The colonies measuring 20-100 μ diameter were detected

after 3 days' incubation, and they reached almost 0.5 mm in diameter by 7-10 days. The colonies did not have a central nipple, but they appeared as convex structures with a granular surface (Goodwin et al., 1967; Friis, 1969). Giemsa-stained touch preparations of colonies revealed pleomorphic mycoplasma-type elements (Goodwin et al., 1965).

Wegmann et al. (1969) reported that M. hyopneumoniae appeared round or oval in shape with a mean diameter of 0.5-0.7 μ under the electron microscope; the organisms were situated mainly between the cilia of the bronchioles and less often within alveoli and in polymorphonuclear leucocytes and alveolar macrophage. In another transmission electron microscopic study, Mebus and Underdahl (1977) found that M. hyopneumoniae appeared oval in shape and was between the cilia rather than on the epithelial cell surface.

Nutrition and Growth

M. hyopneumoniae requires sterols, ferments glucose, but does not hydrolyze urea; tests for phosphatase production and tetrazolium reduction are negative, and tellurite reduction is weakly positive (Goodwin et al., 1967; Friis, 1974a; Jensen et al., 1978; Rose et al., 1979). The organism can utilize 3 gm/l of arginine, but the usual 10 gm/l arginine inhibits the growth of M. hyopneumoniae (Leach, 1976).

Hayflick and Stinebring (1955) first demonstrated that a mycoplasma replicated in tissue culture. Later, the J strain of M. hyopneumoniae was isolated from pneumonic lung tissue of a pig with plasma clot tissue cultures, and the organism was adapted to pig lung monolayer cultures (Betts and Whittlestone, 1963; Goodwin and Whittlestone, 1963). Strain 11 of M. hyopneumoniae was isolated from pneumonic lung of a pig (L'Ecuyer and Switzer, 1963) and cultured in pig monolayer cell cultures (Maré and Switzer, 1966b). Furthermore, L'Ecuyer (1969) recovered 3 Canadian strains of M. hyopneumoniae in pig testicle cell cultures.

L'Ecuyer and Switzer (1963) observed no cytopathogenic effects in Giemsa-stained coverslip preparations of cell cultures inoculated with the agent they were working with. Maré and Switzer (1966b) also reported that no cytopathogenic effect was observed in lung monolayer cell cultures inoculated with strain 11. But, Betts and Whittlestone (1963) found a cytopathogenic effect in plasma clot cultures inoculated with their J strain at 14 days. Goodwin and Whittlestone (1963) reported a mosaic cytopathogenic effect occurred in pig lung monolayer cell cultures subsequent to the appearance of organisms. L'Ecuyer (1969) also detected cytopathogenic effects in pig testicle cell cultures inoculated with Canadian strains. Pijoan (1975) reported

that M. hyopneumoniae caused no cytopathogenic effect although the organisms were seen in pig kidney primary tissue cultures on day 6 postinoculation.

Goodwin and Whittlestone (1964) first cultivated M. hyopneumoniae in nonliving media-tissue culture feeding fluid and boiled pig lung tissue cultures. Subsequently, Maré and Switzer (1966b) developed DPB-BHI medium which contained Dulbecco phosphate buffer, beef-heart infusion, and turkey serum; the opalescence developed and the pH fell from 7.5 to 6.7 after 5 days' incubation. Goodwin et al. (1967) developed A 22 Cambridge liquid medium which contained Hartley's broth. In A 26 liquid medium, a faint opalescence and an obvious pH change occurred after 1 or 2 to 20 days depending on the concentration of inocula (Goodwin et al., 1967; Whittlestone, 1973). L'Ecuyer (1969) developed broth medium A which contained gastric mucin. Goodwin and Hurrell (1970) incorporated M. hyorhinis porcine antiserum in A 26 medium for the primary isolation. Yamamoto et al. (1971) improved the selective medium which contained M. hyorhinis rabbit antiserum and 2 u/ml of kanamycin sulfate for inhibition of the growth of M. hyorhinis.

Friis (1969, 1971b) developed a selective medium for primary isolation of M. hyopneumoniae; thallium acetate 1/10,000 and bacitracin 0.2 mg/ml were added into the medium.

Growth of the organism was promoted by rolling in a revolving drum. Furthermore, Friis (1971c) found that a broth culture containing 5% M. hyorhinis rabbit antiserum and 0.5 mg/ml cycloserine prevented replication of M. hyorhinis; he also mentioned that 0.15 mg/ml of bacitracin and meticillin were incorporated for general bacteriostatic purposes. Later, Friis (1975) recommended a new medium for primary isolation and propagation of M. hyopneumoniae; the basal medium was made from commercial dehydrated products, and 20% swine serum, yeast extract, bacitracin, meticillin, thallium acetate and phenol red were added. Friis (1979) modified his selective medium (1975); equal amounts of horse and swine serum and an acid extract of fresh baker's yeast (Herderscheê, 1963) were added in the basal medium. This medium improved the primary isolation of the slowly growing, acidifying M. hyopneumoniae.

Goodwin (1976) developed an improved selective medium which contained 10% M. hyorhinis rabbit antiserum and 90% A 26 medium; he suggested that 27% serum promoted the growth of M. hyopneumoniae. Wilson (1976) reported that 5% rabbit serum enhanced the color change of medium. Etheridge et al. (1979) developed the MH medium which contained DNA, β-NAD and glucose; this medium improved the primary isolation of the Beaufort strain of M. hyopneumoniae.

Maré and Switzer (1965) first reported growth of

colonies of M. hyopneumoniae on solid medium with 1% agar. Goodwin et al. (1965) found that M. hyopneumoniae colonies grew on A 22 medium with Ionagar No. 2 in a 5-10% CO_2 atmosphere in air. Goodwin and Pryor (1970) mentioned that M. hyopneumoniae might be isolated directly on solid medium from pneumonic lesions in pigs. Friis (1969) prepared the solid medium with 0.9% Noble agar. In 1975, Friis found that the incorporation of DEAE-Dextran (100 mg/100 ml) promoted the growth of M. hyopneumoniae on solid medium with 0.8% Ionagar No. 2 or Agar-Agar.

Survival of M. hyopneumoniae under different conditions was studied by Goodwin (1972). In liquid medium, the organism survived at room temperature for 18-31 days and in the refrigerator for 7-14 weeks. On solid medium, the survival time at 37°C was at least 20 days. In pneumonic tissue, the organism survived at room temperature for 7 days and at least 11 days in the refrigerator. Friis (1973) investigated the survival time of M. hyopneumoniae after drying in air at room temperature. The mean titer of the organism had dropped from 10^6 color changing units (ccu) to 10^2 ccu after 4 days storage, but no viable organisms were detected at 8 days after drying. Friis (1974b) also investigated heat stability of M. hyopneumoniae; in liquid medium, the organism was inactivated after 30 minutes at 45°C and after 2 minutes at 50°C.

Serological Techniques for Identification of Mycoplasma

Growth inhibition test

Priestly (1952) first demonstrated that growth of mycoplasma in liquid medium was inhibited by specific antiserum against the organism. Huijsmans-Evers and Ruys (1956) reported that growth of human genital mycoplasma on solid media was inhibited by antiserum-saturated paper discs. Clyde (1964) modified this technique and identified human mycoplasma isolates on agar medium. Since that time, the growth inhibition test has been used widely to identify strains of M. hyopneumoniae from pneumonic lung of pigs (Goodwin et al., 1967; Friis, 1969, 1971a; Marley et al., 1971; Furlong and Turner, 1975; Etheridge et al., 1979; Tiong, 1981).

Metabolic inhibition test

Jensen (1964) described a quantitative method for measuring the inhibitory effect of antiserum on mycoplasma.

Later, Taylor-Robinson et al. (1966) developed this technique for the quantitative measurement of growth-inhibiting antibody to the acid-producing mycoplasma species. These species were identified by specific antiserum which inhibited the acid shift in the pH of the medium.

Fernald et al. (1967) demonstrated that a heat-labile factor greatly enhanced this test. Strains of M. hyo-

pneumoniae have also been identified with the metabolic inhibition test (Goodwin et al., 1967, 1968; Friis, 1969, 1971a; Yamamoto et al., 1971).

Growth precipitation test

Dinter and Taylor-Robinson (1969) first observed that precipitation lines formed around the antiserum impregnated disc used in the growth inhibition test. Krogsgaard-Jensen (1972) demonstrated that this technique was highly specific and that cross reactions between closely related strains of mycoplasma occurred only after long incubation. Goiš and Kuksa (1975) demonstrated that the growth precipitation test was well-suited for the identification of M. hyorhinis and M. hyopneumoniae. Heitmann and Kirchhoff (1978) reported that a modified growth precipitation test was suitable for typing acholeplasma strains. Ernø and Salih (1980) mentioned that cross reactions were detected within the arginine-positive mycoplasmas and within a group of glucose- and serum digestion-positive species of mycoplasma by this test.

Immunofluorescence

The immunofluorescence technique was used for study of mycoplasmas isolated from primary atypical pneumonia by Liu (1957). Del Giudice et al. (1967) reported that immunofluorescent staining of mycoplasma colonies could be detected

on agar medium. L'Ecuyer and Boulanger (1970) observed good fluorescent intensity of M. hyopneumoniae in smears of broth cultures with the immunofluorescence technique. Jansson (1974) reported that M. hyorhinis strains gave strong immunofluorescence; the colonies did not lose antigenicity during drying. Armstrong (1976) developed a filter membrane-fluorescent antibody method for detection of M. hyopneumoniae; the inoculated membranes were incubated in broth until the pH changed.

Antigenic Heterogeneity of Mycoplasma

Antigenic heterogeneity has been demonstrated among strains of M. hominis with agglutination (Nicol and Edward, 1953), complement fixation and gel diffusion (Taylor-Robinson et al., 1963) and metabolic inhibition (Purcell et al., 1966) tests. Antigenic differences also were detected among M. arthritidis (Lemcke, 1964) and among M. pulmonis strains (Leach and Butler, 1966). Purcell et al. (1967) reported that no significant differences were observed among strains of M. orale type 1, M. orale type 2, M. salivarium or M. hyorhinis with the metabolic inhibition test. Deeb and Kenny (1967) observed that a subtype-specific antigen, the heat-stable complement-fixing and precipitating antigen, existed in M. pulmonis strains.

Taylor-Robinson and Berry (1969) studied 9 strains of M. gallisepticum with the metabolic inhibition test, and detected minor antigenic differences among them. They suggested that the 9 strains belonged to at least 3 subtypes.

No differences were detected between virulent and avirulent strains of M. pneumoniae with complement fixation, metabolic inhibition and immunodiffusion techniques (Lipman et al., 1969). Golightly-Rowland et al. (1970) reported that no distinct differences were observed between virulent and avirulent strains of M. arthritidis with complement fixation and gel diffusion tests, but the slow growing, more virulent strains were less susceptible to inhibition than the avirulent or less virulent strains in the growth inhibition and metabolic inhibition tests.

Cole et al. (1970) reported that human and chimpanzee strains of M. hominis could not be differentiated biochemically but that they could be distinguished by the growth inhibition and metabolic inhibition tests. Del Giudice et al. (1971) found that human and nonhuman primate strains of M. primatum cross reacted in the growth inhibition test, but only minor differences were observed by means of the immunofluorescence test.

Although M. mycoides subsp. mycoides and M. mycoides subsp. capri cross reacted in double diffusion and growth

precipitation tests, serological heterogeneity was detected with the metabolic inhibition test (Al-Aubaidi et al., 1972; Krogsgaard-Jensen, 1972; Ernø and Jurmanová, 1973).

The J strain of M. suipneumoniae was indistinguishable from strain 11 of M. hyopneumoniae with the growth inhibition test (Goodwin et al., 1967; Rose et al., 1979), metabolic inhibition test (Goodwin et al., 1967; Hodges et al., 1969) and the direct fluorescent antibody test (Rose et al., 1979). Roberts and Little (1970) observed antigenic similarity among M. hyopneumoniae strains with the complement fixation test. Friis (1971a) reported that some antigenic differences were observed between the Danish isolates of M. hyopneumoniae and the J strain of M. suipneumoniae in the growth inhibition and metabolic inhibition tests. Jansson (1974) observed that no cross reactions were detected between M. hyorhinis and M. hyopneumoniae with indirect epiimmunofluorescence, but that cross reactions occurred with the growth inhibition, metabolic inhibition and indirect haemagglutination tests. In their study of the growth precipitation test, Goiš and Kuksa (1975) reported no cross reactions between M. hyopneumoniae and M. hyorhinis strains. Friis (1977) investigated a number strains of M. hyopneumoniae and M. flocculare by means of the growth precipitation test, and slight cross reactions were detected

with a few of the laboratory strains towards the end of the observation period.

Dinter and Taylor-Robinson (1969) detected close similarities among strains of M. hyorhinis with the metabolic inhibition test although antigenic heterogeneity within the species was detected with the growth inhibition test. Friis (1971a) found that different strains of M. hyorhinis showed great variation in the growth inhibition and metabolic inhibition tests. Jansson (1974) observed some serological differences among various strains of M. hyorhinis with the growth inhibition, metabolic inhibition, indirect haemagglutination and epiimmunofluorescence techniques. Goiš et al. (1974) reported that 7 strains of M. hyorhinis, isolated in different countries, were compared by the growth inhibition, metabolic inhibition and latex agglutination tests. Antigenic heterogeneity among M. hyorhinis strains was shown by means of all 3 tests, suggesting that the strains belonged to subtypes. Furthermore, Goiš and Kuksa (1975) observed variation in the intensity of reactions obtained with strains of M. hyorhinis in the growth precipitation test. Friis (1976) isolated 7 strains of M. hyorhinis from the conjunctiva of swine, all strains were glucose and phosphatase positive, and all strains represented a distinct serogroup with the growth

inhibition, metabolic inhibition, indirect immunofluorescence and immunodiffusion tests. The 7 strains might be regarded as a new subspecies of M. hyorhinis.

A close antigenic relationship among isolates of M. hyosynoviae was revealed with growth inhibition (Friis, 1970; Goiš and Taylor-Robinson, 1972; Ross and Karmon, 1970) and metabolic inhibition tests (Ross and Karmon, 1970). Goiš and Taylor-Robinson (1972) observed that the metabolic inhibiting titer of antiserum against strain A 40 was about 10-fold higher with the homologous culture than against strains A 26 and Ha 32. They also observed a close serological relationship between strain A 40 and strain S 16 (Ross and Karmon, 1970) and M 60 (Friis, 1970) with the growth inhibition and metabolic inhibition tests. Ross et al. (1978) studied the serological characteristics of 5 strains of M. hyosynoviae isolated from joints, a lymph node, and pharyngeal secretions of swine; antigenic heterogeneity and type-specificity among the strains were revealed with the growth inhibition, metabolic inhibition and direct epiimmunofluorescence techniques. With the metabolic inhibition test, a great difference between homologous and heterologous strains was observed, as great as 32-fold differences in titer among 5 strains.

Electrophoretic Methods

Polyacrylamide gel electrophoresis

Fowler et al. (1963) found that each mycoplasma had a characteristic protein pattern with starch gel electrophoresis. Rottem and Razin (1967) and Razin and Rottem (1967) developed polyacrylamide gel electrophoresis according to Davis (1964) and Takayama et al. (1966) for the study of electrophoretic patterns of mycoplasma cell proteins; polyacrylamide gel electrophoresis was much superior to starch gel electrophoresis for the separation of proteins. They suggested that this method proved the identity and dissimilarity of mycoplasma strains and was suited for the identification and classification of other microorganisms. Ackroyd (1967) improved this technique with the flat gel system which was convenient for the comparison of many samples. Later, Boden and Kirchhoff (1977) developed a horizontal acidic polyacrylamide flat gel electrophoresis, and the gels were prepared between glass plates prior to insertion in the electrophoretic chamber.

Zola et al. (1970) reported that no difference existed between electrophoretic patterns of proteins from M. gallisepticum X 95 prepared from different batches, grown in different media and grown for different periods of time. In a study of porcine mycoplasmas, Wreghitt et al. (1974)

reported that the amount of protein in the extract and the age of the culture did influence the electrophoretic patterns and bands. They were in agreement with Armstrong and Yu (1970) and Rosendal (1973) with respect to influence of age of culture.

Rottem et al. (1973) reported that during growth, a large amount of serum albumin was bound to Acholeplasma laidlawii cell membranes when the pH reached 5 and below. Yaguzhinskaya (1976) detected serum proteins in preparations of mycoplasma cell proteins by means of polyacrylamide gel electrophoresis; he found that contamination of mycoplasma cell proteins was mainly due to serum proteins aggregated together with mycoplasma cells during centrifugation rather than to adsorption of serum proteins on the cell surface. Paroz and Nicolet (1978) reported that polyacrylamide gels of mycoplasma proteins contained periodic acid-Schiff (PAS) positive bands which might have originated from the growth medium. Nicolet et al. (1980) observed that polyacrylamide gels of glycoproteins of M. hyopneumoniae (75,000 M.W.) and M. hyorhinis (80,000 M.W.) contained PAS positive bands. The extracts were prepared by lithium diiodosalicylate solubilization and aqueous phenol extraction. They also observed that a serum glycoprotein contaminated the glycoprotein preparations of M. hyopneumoniae grown in the

presence of swine serum.

Two-dimensional immunoelectrophoresis

Ressler (1960) first described the principle of crossed antigen-antibody electrophoresis. Subsequently, Laurell (1965) modified the technique and developed a second electrophoretic run of the protein fractions after transfer to an antibody-containing agar. Johansson and Hjertén (1974) reported that as many as 20 components of Acholeplasma laidlawii membrane proteins were resolved with Tween 20 by this method. They recommended use of two-dimensional immunoelectrophoresis for qualitative and quantitative analysis of membrane fractions. Thirkill and Kenny (1974) incorporated 0.5% Triton X-100 in both dimensions for maintaining solubility of the components and avoiding distortion of peaks. In their study of arginine-utilizing mycoplasmas, 9-20 antigenic components of mycoplasma membrane proteins were resolved by this method. Thirkill and Kenny (1975) demonstrated that two-dimensional immunoelectrophoresis was suitable for antigenic analysis of mycoplasma. By use of enhancement and suppression techniques, the immunologic identity and uniqueness of components were established without fractionation of the organisms. Helenius and Simons (1977) reported that incorporation of a charged detergent with proteins solubilized in a nonionic detergent enhanced

the mobility of amphophilic proteins. Wroblewski et al. (1977) reported that deoxycholate, Triton X-100 and Tween 20 solubilized Acholeplasma laidlawii membranes effectively, and that deoxycholate solubilized Spiroplasma citri membranes. Alexander and Kenny (1978) reported that the incorporation of 0.5% Triton X-100 and 0.1% sodium deoxycholate in the agarose for the first phase of electrophoresis greatly enhanced the anodic migration and resolution of membrane antigens of M. arginini in two-dimensional immunoelectrophoresis.

Two-dimensional immunoelectrophoresis has the high sensitivity and resolution needed for antigenic analysis, but this method is restricted to antigens which are solubilized by detergent and have an electrophoretic charge (Kenny, 1979).

Electrophoretic Heterogeneity of Mycoplasma

Rottem and Razin (1967) observed differences in electrophoretic patterns among strains of M. mycoides subsp. mycoides and M. mycoides subsp. capri. Razin and Rottem (1967) examined the close similarity of the electrophoretic patterns between M. hominis type 2 strains and M. arthritidis. Zola et al. (1970) reported polyacrylamide gel electrophoresis indicated that M. granularum strain M 305/68 was very similar

to M. laidlawii strains. Wreghitt et al. (1974) demonstrated clear differences between various porcine mycoplasma species, and some differences among M. hyorhinis strains by electrophoresis in polyacrylamide gel. Goiš et al. (1974) found that, although antigenic heterogeneity was detected easily among 7 strains of M. hyorhinis by several serologic techniques, no differences were observed among the strains following electrophoresis in polyacrylamide gel. Boden and Kirchhoff (1977) observed marked differences of electrophoretic patterns obtained with different acholeplasma species by horizontal polyacrylamide flat gel electrophoresis. Ross et al. (1978) studied electrophoretic patterns of 5 strains of M. hyosynoviae proteins; the same number and spacing of peaks were observed in electropherograms, but band intensity varied. No differences of electrophoretic patterns were observed between batches and among cultures grown in different media. Rose et al. (1979) reported that the electrophoretic patterns of strain 11 of M. hyopneumoniae and strain J of M. suipneumoniae were identical, and the similarities and differences of the gel patterns were observed between strain Ms 42 of M. flocculare and strains 11 and J by polyacrylamide gel electrophoresis. Using polyacrylamide gel electrophoresis, Texier et al. (1980) detected certain proteins that were synthesized only

by a virulent strain of M. pulmonis but not by a non-pathogenic strain.

Rodwell and Rodwell (1978) compared the protein patterns of small colony (SC) and large colony (LC) types of M. mycoides subsp. mycoides and M. mycoides subsp. capri by two-dimensional gel electrophoresis; the LC strains were more closely related to M. mycoides subsp. capri than to the SC strains of M. mycoides subsp. mycoides. A significant congruence was detected between the protein patterns of the strains within M. mycoides subsp. mycoides SC type, M. mycoides subsp. mycoides LC type or M. mycoides subsp. capri. Furthermore, Archer (1979) reported that SC and LC strains of M. mycoides subsp. mycoides and M. mycoides subsp. capri shared some antigenic proteins. These were demonstrated by immunoprecipitation of Triton X-100-solubilized proteins that had been separated by two-dimensional gel electrophoresis.

Hansen et al. (1979b) compared the protein composition of the virulent and avirulent M 129 strains of M. pneumoniae by one or two-dimensional gel electrophoresis. In polyacrylamide gel electrophoresis, a single high-molecular-weight protein presented in the virulent strain but not in the avirulent strain. In two-dimensional gel electrophoresis, 3 proteins were identified in the virulent strain but not in

the homologous avirulent strain. In another study, Hansen et al. (1979a) detected a high-molecular-weight protein in virulent M 129 strain and hemadsorption-negative mutant strain HA 1 of M. pneumoniae but not in the mutant strain HA 2 by means of polyacrylamide gel electrophoresis. Subsequently, using isoelectric focusing in sodium dodecyl sulfate polyacrylamide gels, Hansen et al. (1981) reported that the mutant HA 1 strain of M. pneumoniae possessed a protein spot, α-HA 1, but lacked the virulent strain-specific protein A; and mutant strain HA 2 possessed protein spot, α-HA 2, and the virulent strain-specific protein A. Significant differences of gel patterns between mutant HA 1 and both the virulent strain and mutant strain HA 2 were resolved; mutant strain HA 1 exhibited a protein spot, α-HA 1, but lacked virulent strain-specific proteins B and C. The protein spot patterns were identical between mutant strain HA 2 and the virulent strain by electrophoresis in a non-equilibrium pH gradient sodium dodecyl sulfate-polyacrylamide gel.

Thirkill and Kenny (1974) found that 3 strains of M. arginini showed strong cross reactions but also significant antigenic differences between strains by two-dimensional immunoelectrophoresis. The similarities were shown between M. arginini and M. gateae, but no similarities were observed between nonglycolytic arginine-utilizing mycoplasma species

and M. gallisepticum by this method. Furthermore, Thirkill and Kenny (1975) studied the species-specific and strain-specific antigens in 3 strains of M. arginini by two-dimensional immunoelectrophoresis. Three common components were observed in all strains; unique fast electrophoretic components were shown in strain G-230 and strain 23243, and 4 unique antigens were detected by enhancement and suppression techniques. Alexander and Kenny (1977) used two-dimensional immunoelectrophoresis to identify membrane and cytoplasmic antigens of M. arginini. Four cytoplasmic and 2 common membrane antigens were found in all 3 strains, and a complex unique membrane antigen was found in strain G-230.

Wroblewski and Ratanasavanh (1976) reported that one common antigen was observed between Acholeplasma laidlawii and Acholeplasma granularum by this method. Lee and Kenny (1980) detected superoxide dismutase activity in lysates of acholeplasma species in agarose-gel electropherograms or in rocket immunoelectropherograms. The superoxide dismutase from different acholeplasma species exhibited serological and electrophoretic heterogeneity.

MATERIALS AND METHODS

Mycoplasma Strains

In this study, 6 field strains of M. hyopneumoniae and 4 mycoplasma reference strains were compared by means of serological and electrophoretic techniques. The source and history of the mycoplasma strains are outlined in Table 1.

The field strains of M. hyopneumoniae were isolated by Dr. R. F. Ross and cloned 3 times by Ms. Barbara J. Zimmermann; the isolation and colonization procedures were as follows. Pig lungs with lesions of mycoplasmal pneumonia collected at necropsy at the Veterinary Medical Diagnostic Laboratory, Iowa State University, Ames, Iowa, were sent to this laboratory for isolation and identification of mycoplasma. About 1 g of lung tissue including healthy and diseased lobules was ground in a Ten-Broeck tissue grinder, suspended in 5 ml of Friis broth medium without antibiotics, and followed by serial 10-fold dilutions to 10^{-7} in Friis broth medium with antibiotics in a 2-ml system. The broth culture tubes were rolled at 37°C in a drum until the pH changed. Suspect growth was subcultured to Friis solid medium. Following incubation in a candle jar for 3-10 days, plates were examined for colonies. Identification of colonies was achieved by means of epiimmunofluorescence. For purification of each field strain, the broth culture was filtered through a 0.45 μ

Table 1. Identity and origin of the mycoplasma strains

Mycoplasma strain		Source of strain (reference)	Strain supplied by
M. hyopneumoniae	11	Maré and Switzer (1965)	Dr. W. P. Switzer Veterinary Medical Research Institute, Iowa State University, Ames, Iowa, USA
	1361A	Isolated from mycoplasmal pneumonic lung of pigs by Dr. R. F. Ross and Ms. Barbara J. Zimmermann	Ms. Barbara J. Zimmermann, Veterinary Medical Research Institute, Iowa State University, Ames, Iowa, USA
	1375C	"	"
	1417	"	"
	1419	"	"
	1424B	"	"
	1472C	"	"
	J	Goodwin et al. (1965)	American Type Culture Collection, 1203 Parklawn Drive, Rockville, Maryland, USA
M. flocculare	Ms 42	Meyling and Friis (1972)	Dr. D. L. Rose National Institute of Allergy and Infectious Diseases, Bethesda, USA
M. hyorhinis	BTS 7	Switzer (1955)	Dr. W. P. Switzer

filter membrane[1], and serial 10-fold dilutions of the filtrate were made in Friis broth medium to 10^{-8}; 1 drop of 10^{0}, 10^{-1}, 10^{-2} and 10^{-3} dilutions was plated on Friis solid medium and incubated at 37°C in a candle jar. One isolated colony was picked from the plate with a Pasteur pipette under a stereomicroscope[2], and the colony was reinoculated into 1.8 ml of Friis broth medium. The filtration-cloning procedure was repeated 3 times, then the purified broth cultures of M. hyopneumoniae were kept at -70°C.

Culture Media

Friis broth medium: This medium was prepared basically as described by Friis (1975). It consisted of modified Hanks' balanced salt solution, 500 ml; distilled water, 750 ml; Bacto brain heart infusion[3], 8.2 g; Bacto PPLO broth w/o CV[3], 8.7 g; yeast[4] extract, 60 ml; 0.2% phenol red[5] solution, 9 ml; bacitracin[6], 250 mg; meticillin[6], 250 mg; thallium acetate[7], 0.1 mg; and heated horse or swine serum,

[1]Gelman Instrument Company, Ann Arbor, Michigan.

[2]Bausch and Lomb Inc., Rochester, New York.

[3]Difco Laboratories, Detroit, Michigan.

[4]Fleischmann's pure dry yeast, type 20-40, Standard Brands Inc., New York.

[5]Eastman Organic Chemicals, Rochester, New York.

[6]Sigma Chemical Company, St. Louis, Missouri.

[7]Fisher Scientific Company, Fairlawn, New Jersey.

440 ml. After the pH was adjusted to 7.4, the medium was sterilized by Seitz filtration. In an alternative Friis broth medium, heated horse serum was replaced by 25% unheated horse serum and 1% dextrose, and the pH was adjusted to 7.6; this medium was used for the metabolic inhibition test. For solid medium, 0.8% Ionagar No. 2[1] and 10 mg/100 ml DEAE-Dextran[2] were added to Friis broth medium.

Rabbit muscle infusion broth medium: This medium was prepared according to the procedure described by Friis (1977). Rabbit muscle, 125 g, was blended with 1 l of distilled water in a blender, and the suspension was infused at 4°C overnight. The infusion was heated in a water bath to 93-95°C for 30 minutes and stirred about 5 minutes, then the broth was cooled and filtered through 2 single thicknesses of gauze. Ten g of Bacto peptone[3] and 5 g of NaCl were added to the filtrate, and the pH was adjusted to 7.8 with 1 N NaOH. The broth was heated again in a water bath to 93-95°C for 3 minutes and filtered through a Whatman No. 1 filter paper. Rabbit muscle infusion broth medium contained rabbit muscle infusion, 300 ml; modified Hanks' balanced salt solution, 200 ml; yeast extract,

[1]Oxoid Limited, London, England.

[2]Sigma Chemical Company, St. Louis, Missouri.

[3]Difco Laboratories, Detroit, Michigan.

25 ml; 10% dextrose, 10 ml; bactracin, 100 mg; meticillin, 100 mg; 0.2% phenol red, 4.5 ml; and heated rabbit serum, 107 ml. The pH was adjusted to 7.6 and sterilized by Seitz filtration.

Preparation of Antigens and Immunogens

Cell extracts

Cell extracts for polyacrylamide gel electrophoresis were prepared according to the method reported by Razin and Rottem (1967). Each strain was cultured in 200 ml of Friis broth medium containing 20% heated swine serum inoculated with 5 ml of actively growing broth culture. The inoculated broth culture was shaken at 50 rpm/min in a water bath[1] for 72 hours at 37°C. Cells were sedimented and washed 2 times in 0.25 M NaCl by centrifugation[2] at 10,000 rpm for 20 minutes at 4°C. Cells were resuspended in 1 ml of 0.25 M NaCl. The protein concentrations of extracts were measured by the Folin phenol[3] method of Lowry et al. (1951) with Lab-trol[4] as a standard and adjusted to

[1]AquaTherm water bath shaker, New Brunswick Scientific Co., Inc., Edison, New Jersey.

[2]Sorvall superspeed RC 2-B, Ivan Sorvall Inc., Newtown, Connecticut.

[3]Fisher Scientific Company, Fairlawn, New Jersey.

[4]Dade Diagnostics, Inc., Miami, Florida.

2 mg/ml. After the cells had been centrifuged, 0.2 ml of phenol-acetic acid-water (2:1:0.5, w/v/v) was added per mg of cell protein in the pellet and incubated for 30 minutes at 37°C. Cell protein solutions in phenol-acetic acid-water were stored at 4°C until used.

Antigens for two-dimensional immunoelectrophoresis were prepared as described by Thirkill and Kenny (1974). Each strain was grown in 500 ml of Friis broth medium containing 20% heated horse serum inoculated with 10 ml of a 24 hour broth culture. These cultures were shaken at 50 rpm/min in a water bath for 4-6 days at 37°C until the pH changed to 6.8. Cells were harvested and washed 3 times in 20 ml of 0.15 M NaCl with 0.005 M N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid[1] by centrifugation at 10,000 rpm for 20 minutes at 4°C, and the cells were resuspended in 1 ml of distilled water. Cells were disrupted using a Bronwill sonicator[2] at maximum power for 2 minutes with interruptions and an ice bath to prevent overheating. The protein concentration of antigens was estimated by the method of Lowry et al. (1951) and adjusted to 10 mg/ml. The sonicated antigens were stored at -70°C until used.

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[1]Eastman Organic Chemicals, Rochester, New York.

[2]Bronwill Scientific, Rochester, New York.

Immunogens

Each strain was grown in 2 l of rabbit muscle infusion broth. Inoculum consisted of 60 ml of a 24 hour culture which had been grown in Friis broth medium containing 25% heated swine serum. The inoculated broth was incubated in a shaking water bath for 3-5 days at 37°C until the pH changed to 6.8. Organisms were sedimented and washed 3 times in Hanks' balanced salt solution[1] by centrifugation at 10,000 rpm for 20 minutes at 4°C. The organisms were resuspended in 20 ml of Hanks' balanced salt solution and the number of color changing units was determined by serial 10-fold dilutions to 10^{-13} in Friis broth medium. Then 4 ml of 0.9% formalin was added to achieve a 0.15% final concentration and the cell suspensions were left at 4°C overnight. The killing effect of formalin was confirmed by serial 10-fold dilutions in Friis broth medium. The immunogens were stored at -70°C.

Hyperimmune Sera

Antisera to each mycoplasma strain were prepared in 2 New Zealand white rabbits. Rabbits were immunized by 5 intramuscular injections of a mixture of equal amounts of

[1]Grand Island Biological Company, Grand Island, New York.

Freund's complete adjuvant[1] and immunogen; 1.5 ml of the immunogen-adjuvant mixture was given at intervals of 3 days. Three weeks later, the rabbits were given 2 intravenous injections of 1.5 ml of immunogen at 5 day intervals. A test bleeding was obtained prior to the last injection, and antibody titers were determined by the growth inhibition, growth precipitation, metabolic inhibition and tube agglutination (Pijoan and Boughton, 1974) tests. The rabbits were bled from the heart within 10 days of the last immunization.

Antiserum to bovine serum albumin was produced in a rabbit as described by Thirkill and Kenny (1974). Five mg of bovine serum albumin[2] was emulsified in an equal volume of Freund's incomplete adjuvant[1] and the mixture was injected intramuscularly. Three weeks later, 4 intravenous injections were administered at 5 day intervals with 0.5 mg, 1.0 mg, 1.5 mg and 2.0 mg of bovine serum albumin, respectively. The rabbit was bled on the 7th day after the last immunization.

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[1]Miles Laboratories Inc., Naperville, Illinois.

[2]Reheis Chemical Company, Phoenix, Arizona.

Serological Tests

Growth inhibition test

This test was carried out as described by Clyde (1964). Filter-paper discs[1] 5 mm in diameter were soaked with 0.02 ml of antiserum and dried overnight under ultraviolet light. Discs impregnated with normal rabbit serum were used as controls. For mycoplasma cultivation, 20 ml of Friis solid medium was poured into each Petri dish (8.5 cm). Agar plates were inoculated with 0.10 ml of 24 hour Friis broth cultures of M. hyopneumoniae or M. flocculare in dilutions of 10^{-1} and 10^{-2}, or M. hyorhinis in dilutions of 10^{-3} and 10^{-4}. The inoculum was streaked with a bent Pasteur pipette. Discs were slightly pressed on the inoculated agar surface, and the plates were incubated for 4-10 days at 37°C in a candle jar. The results were read with a low-power stereomicroscope, and the inhibition zones were measured from the disc edge to the commencement of growth. Clear zones greater than 1.0 mm were regarded as evidence of growth inhibition activity.

Metabolic inhibition test

This test was done in microtiter plates[2] according to the method described by Taylor-Robinson et al. (1966).

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[1] Difco Laboratories, Detroit, Michigan.

[2] Model IS-MVC-96, Linbro Chemical Co., New Haven, Connecticut.

Dilutions of 24 hour broth cultures of M. flocculare or M. hyopneumoniae to 10^{-2} and 10^{-3} or M. hyorhinis to 10^{-3} and 10^{-4} were used as seed cultures. Rabbit antisera to each mycoplasma strain were filtered through a 0.45 μ membrane filter and inactivated at 56°C for 30 minutes prior to use. Firstly, 0.025 ml of broth medium was added to each well of the microtiter plate with a dropper, then serial 2-fold dilutions of antiserum were made with a diluter. Then, 0.05 ml of seed culture and 0.125 ml of broth medium were added to each well. Antigen control consisted of 0.05 ml of seed culture and 0.15 ml of broth medium; endpoint control contained 0.2 ml of broth medium, pH 6.8; medium control contained 0.2 ml of broth medium, pH 7.6. After the plates were sealed with plate sealers[1] and left at 4°C overnight, they were incubated at 37°C under aerobic conditions until the pH of the antigen control changed to 6.8. The highest dilution of antiserum that prevented a 0.5 pH unit change was recorded as the metabolic inhibiting titer. The results were recorded twice daily by placing the plates over a mirror with a fluorescent white light above the plates.

[1]Cooke Engineering Company, Alexandria, Virginia.

Electrophoretic Methods

Polyacrylamide gel electrophoresis

The electrophoretic techniques were followed as described by Razin and Rottem (1967). Stock solutions of polyacrylamide gel were prepared as reported by Boden and Kirchhoff (1977). Stock solution A contained acrylamide[1], 60 g; N,N'-methylenebisacrylamide[1], 1.6 g; urea[2], 144.14 g; glacial acetic acid, 280 ml; and distilled water, 600 ml. Stock solution B consisted of ammonium persulfate[3], 3 g; urea, 120 g; and distilled water, 200 ml. Gel solution was composed of 30 ml of stock solution A, 10 ml of stock solution B and 0.2 ml of N,N,N',N'-tetramethylethylenediamine[1]. Following mixing, 1.5 ml of the solution was immediately added to each glass tube (5 x 75 mm) with a Pasteur pipette, then each gel was overlayered with 0.5 ml of 75% acetic acid. Polymerization of the acrylamide was carried out at room temperature overnight. One hundred µl of cell protein solution in phenol-acetic acid-water, 50 µl of a 40% sucrose solution in 35% acetic acid and a trace amount of 1% Pyronin Y[1] were mixed well and put on top of the gel. After 0.5 ml of 75% acetic acid had been gently layered over

[1]Eastman Organic Chemicals, Rochester, New York.

[2]J. T. Baker Chemical Co., Phillipsburg, New Jersey.

[3]E-C Apparatus Corp., Philadelphia, Pennsylvania.

the mixture, the tube was filled to the top with 10% acetic acid. Upper and lower reservoirs of the electrophoresis apparatus[1] were filled with 10% acetic acid, then the cationic system of polyacrylamide gel electrophoresis was carried out at room temperature about $2\frac{1}{2}$ hours at 5 ma per tube, until the indicator dye reached the bottom of the gel. Gels were stained for 60 minutes at 37°C with 0.25% Coomassie brilliant blue G-250[2] in methanol-acetic acid-water (5:1:5, v/v/v) after electrophoresis, then the gels were destained for 15 minutes at 37°C in solvent without dye. After decolorization, the gels were washed in 7% acetic acid at 4°C for 48 hours.

Two-dimensional immunoelectrophoresis

The procedures were carried out in a macro-technique or a micro-technique; and barbital buffer, and solutions for protein fixation, staining and destaining were prepared as described by Weeke (1973a, 1973b). The first dimension agarose gels consisted of 0.5% Agarose-M[3] in pH 8.6 barbital buffer, ionic strength 0.05, with 0.5% Triton X-100[4] and 0.1%

[1]Buchler polyanalyst, Fort Lee, New Jersey.

[2]Accurate Chemical and Scientific Corp., Hicksville, New York.

[3]LKB Biochemo Ltd., Bromma, Sweden.

[4]Rohm and Haas Inc., Philadelphia, Pennsylvania.

sodium deoxycholate[1] as described by Thirkill and Kenny (1974) and Alexander and Kenny (1978). The second dimension gels contained 1.0% Agarose-M in pH 8.6 barbital buffer, ionic strength 0.02, as described by Weeke (1973a).

Macro-technique: Each glass plate[2] (84 x 94 mm) placed on a horizontal table[2] was poured with 15 ml of agarose gel containing detergents, then 3 wells (4 mm in dia.) were punched in the gel using a gel puncher[2] and a template[2]. Ten µl of the sonicated antigen, 5 µl of 1 mg/ml bovine serum albumin and a trace amount of 1% bromphenol-blue[1] were added to the well with constriction micropipettes. The plates were placed on the cooling plate[2] of the electrophoresis apparatus[3]. The electrophoresis chambers were filled with pH 8.8 high-resolution buffer[4], ionic strength 0.1, and the power supply[5] was connected to the terminals on the chamber. Electrophoresis in the first dimension was carried out at 10°C, the cooling plate being connected to a LKB 2209 Multitemp cooling unit, for 2 hours at 6-8 v/cm, and the

[1]Fisher Scientific Company, Fairlawn, New Jersey.

[2]LKB Biochemo Ltd., Bromma, Sweden.

[3]LKB 2117 Multiphor, LKB Biochemo Ltd., Bromma, Sweden.

[4]Gelman Instrument Company, Ann Arbor, Michigan.

[5]Model 500 power supply, Bio-Rad Laboratory Ltd., Ontario, Canada.

potential drop was checked with a voltage probe.[1] Each plate was divided into 3 slabs by means of a knife and the template[1], then each slab was transferred to a glass plate (84 x 94 mm) placed on a horizontal table. The antibody-containing agarose which contained 10.5 ml of 1% agarose, 0.5 ml of mycoplasma antiserum and 0.2 ml of anti-bovine albumin rabbit serum was poured onto the remaining area, and electrophoresis in the second dimension was carried out at 10°C for 20 hours at 3 v/cm. Following electrophoresis, the plates were pressed, washed in 0.1 M NaCl and in distilled water, fixed in protein fixation solution for 10 minutes, immersed into 95% ethanol for 5 minutes and dried with a hair dryer. The plates were stained with 0.5% Coomassie brilliant blue G-250 in ethanol-acetic acid-water (4.5:1:4.5, v/v/v) for 30 minutes then destained in solvent alone for 10 minutes. After decolorization, the plates were washed in 0.1 M NaCl at 4°C overnight then washed in distilled water for 15 minutes before drying.

Micro-technique: Glass plates (84 x 94 mm) were covered with 15 ml of agarose containing detergents on a horizontal table, and 5 wells (4 mm in dia.) were punched with a gel puncture[1] and a template[1]. Five µl of the sonicated antigen and 2 µl of 1 mg/ml bovine serum albumin were added to each

[1]LKB Biochemo Ltd., Bromma, Sweden.

well, then first dimension electrophoresis was carried out at 10°C for 75 minutes at 6 v/cm. Following electrophoresis, the plate was divided into 5 slabs with a knife and the template, and each slab was transferred to a glass plate[1] (50 x 75 mm) on a horizontal table. Then, 5 ml of 1% agarose, 0.25 ml of mycoplasma antiserum and 0.1 ml of anti-bovine albumin serum were thoroughly mixed and poured onto the cleared area of the plate. Second dimension electrophoresis was carried out at 10°C for 15-20 hours at 2-3 v/cm. Finally, the gels were pressed, washed, fixed, pressed, dried, stained, destained, washed and dried as described previously.

Horse serum and swine serum were electrophoresed against each anti-mycoplasma rabbit serum as controls.

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[1]American Hospital Supply Corporation, Evanston, Illinois.

RESULTS

Serological Studies

Growth inhibition test

All mycoplasma strains were tested against antisera to 6 field strains of M. hyopneumoniae, strain 11 of M. hyopneumoniae, J strain of M. hyopneumoniae, Ms 42 strain of M. flocculare and BTS 7 strain of M. hyorhinis. The growth inhibition zones shown in Table 2 were calculated from the average width of zones obtained in 2 replicate tests.

All the field strains of M. hyopneumoniae were inhibited by antisera to reference strains 11 and J. The inhibition zone size varied from strain to strain, and the range was between 4.0 and 9.0 mm. It was apparent that strains 1361 A, 1375 C, 1417 and 1472 C of M. hyopneumoniae and J strain of M. hyopneumoniae were inhibited most effectively by their homologous antisera. Also, antiserum to strain 1361 A inhibited the heterologous strains effectively; the range of inhibition zone size was between 6.5 and 9.0 mm. A close reciprocal relationship was shown between strain 11 of M. hyopneumoniae and J strain of M. hyopneumoniae.

Strain Ms 42 of M. flocculare and BTS 7 strain of M. hyorhinis were inhibited by their homologous antisera; and no cross reactions were apparent between mycoplasma

Table 2. Sensitivity of mycoplasma strains to rabbit antisera in the growth inhibition test

Strain		Antiserum									
		11	1361A	1375 C	1417	1419	1424 B	1472 C	J	Ms 42	BTS 7
M. hyopneumoniae	11	6.0[a]	6.5	5.0	5.5	4.5	4.5	5.5	5.5	0	0
M. hyopneumoniae	1361 A	5.0	9.0	5.5	5.5	5.0	6.0	6.5	6.5	0	0
M. hyopneumoniae	1375 C	7.0	8.5	8.5	7.5	6.0	6.0	6.0	7.0	0	0
M. hyopneumoniae	1417	6.5	8.5	7.0	9.0	6.0	6.5	6.0	7.0	0	0
M. hyopneumoniae	1419	6.0	8.5	6.0	7.5	6.5	6.5	5.5	6.5	0	0
M. hyopneumoniae	1424 B	5.0	8.5	6.0	5.5	4.0	7.0	4.5	5.0	0	0
M. hyopneumoniae	1472 C	7.0	9.0	7.5	7.5	6.0	7.5	9.0	7.0	0	0
M. hyopneumoniae	J	6.0	7.0	4.5	5.5	4.0	5.5	5.0	7.0	0	0
M. flocculare	Ms 42	0	0	0	0	0	0	0	0	6.0	0
M. hyorhinis	BTS 7	0	0	0	0	0	0	0	0	0	3.5

[a]Average zone size (mm) calculated from 2 replicate tests.

species.

No zones of inhibition were detected around discs impregnated with normal rabbit serum.

Metabolic inhibition test

In metabolic inhibition tests, antisera prepared against each mycoplasma strain were tested against all the field and reference strains. The metabolic inhibition titers given in Table 3 were the average of 2 replicate tests.

All field strains of M. hyopneumoniae were inhibited to significant titers by antisera to strain 11 of M. hyopneumoniae and J strain of M. hyopneumoniae. The range of homologous titers was between 128 and >16,384, whereas heterologous titers ranged between 64 and >16,384. The metabolism of strains 1361 A, 1417, 1472 C and J was inhibited most effectively by the homologous antisera. As in the growth inhibition test, the homologous titers were commonly greater than heterologous titers. But, the metabolism of strains 1419 and 1424 B was inhibited to lower titers by the homologous and heterologous antisera.

Cross reactions occurred between strain 1419 of M. hyopneumoniae and antiserum to Ms 42 strain of M. flocculare, and between Ms 42 strain of M. flocculare and antisera to strains 1361 A, 1417 and 1419 of M. hyopneumoniae. The metabolism of BTS 7 strain of M. hyorhinis was inhibited only by its

Table 3. Sensitivity of mycoplasma strains to rabbit antisera in the metabolic inhibition test

Strain		Antiserum									
		11	1361 A	1375 C	1417	1419	1424 B	1472 C	J	Ms 42	BTS 7
M. hyopneumoniae	11	4,096[a]	8,192	1,024	2,048	512	1,024	1,024	1,024	<16	<16
M. hyopneumoniae	1361 A	128	2,048	512	1,024	128	512	512	256	<16	<16
M. hyopneumoniae	1375 C	256	512	256	512	128	256	128	256	<16	<16
M. hyopneumoniae	1417	2,048	4,096	2,048	>16,384	2,048	2,048	4,096	4,096	<16	<16
M. hyopneumoniae	1419	1,024	>16,384	2,048	4,096	1,024	2,048	2,048	2,048	32	<16
M. hyopneumoniae	1424 B	256	512	64	256	64	128	128	64	<16	<16
M. hyopneumoniae	1472 C	1,024	2,048	1,024	2,048	1,024	4,096	8,192	8,192	<16	<16
M. hyopneumoniae	J	4,096	4,096	2,048	8,192	1,024	2,048	1,024	8,192	<16	<16
M. flocculare	Ms 42	<16	128	<16	32	16	<16	<16	<16	2,048	<16
M. hyorhinis	BTS 7	<16	<16	<16	<16	<16	<16	<16	<16	<16	>16,384

[a]Average metabolic inhibition titer of 2 replicate tests expressed as reciprocal of highest antiserum dilution that prevented a 0.5 pH unit change.

homologous antiserum; and none of the M. hyopneumoniae strains or the Ms 42 strain of M. flocculare were inhibited by antiserum to the BTS 7 strain of M. hyorhinis.

The metabolism of the mycoplasma strains was not inhibited by normal rabbit serum.

Electrophoretic Studies

Polyacrylamide gel electrophoresis

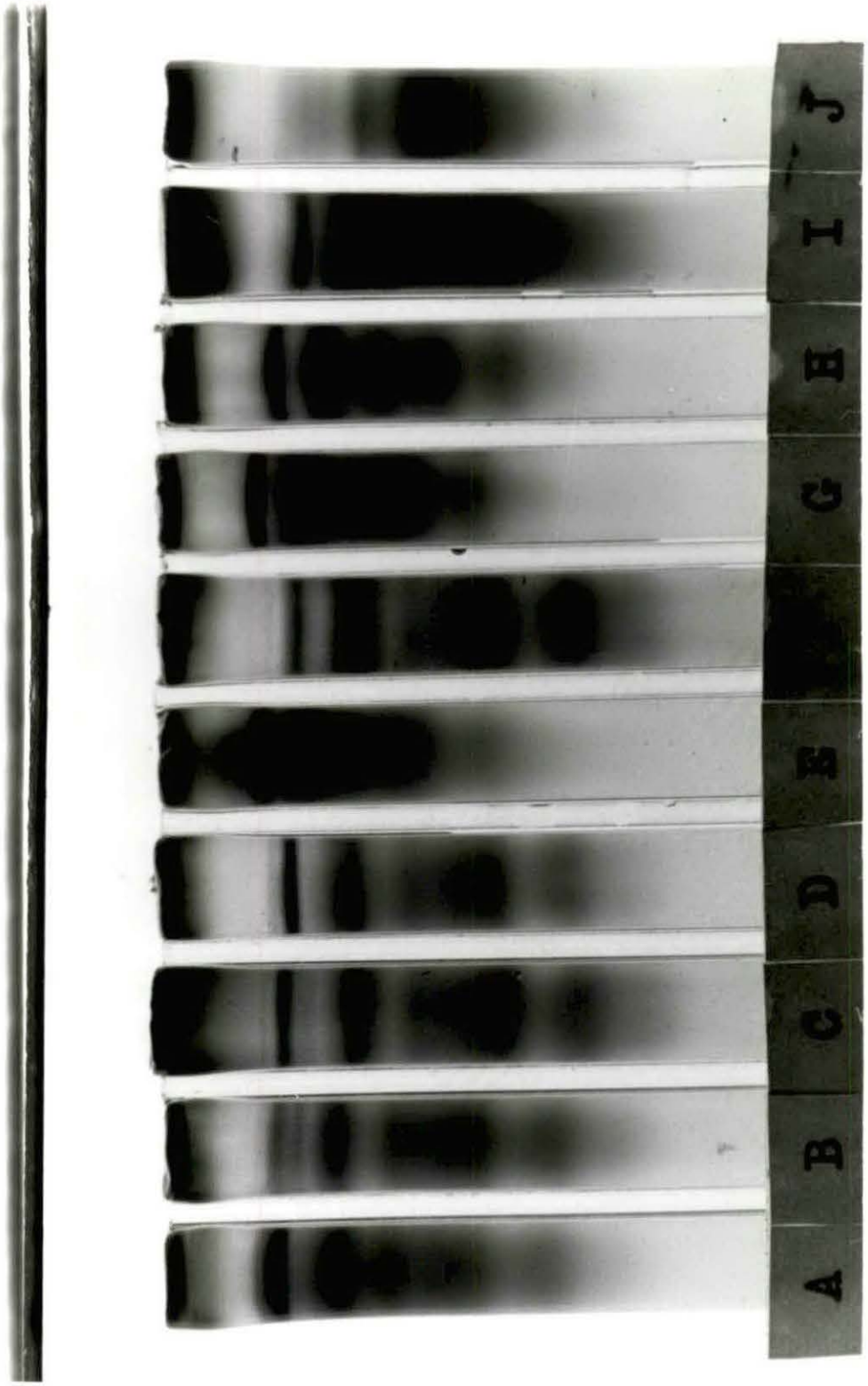
Electrophoretic patterns obtained in polyacrylamide gels with different batches of cell proteins from all mycoplasma strains were highly reproducible.

Patterns in Figure 1 reveal some common as well as distinctive bands between the 3 different mycoplasma species. The gel pattern of Ms 42 strain of M. flocculare was similar to the patterns of M. hyopneumoniae strains, but the pattern of BTS 7 strain of M. hyorhinis differed markedly from those of the M. hyopneumoniae strains and Ms 42 strain of M. flocculare.

Although the patterns of the field and reference strains of M. hyopneumoniae shared the majority of bands, minor differences were observed in the top one-third of the gels. The pattern of strain 1424 B lacked 2-3 weak bands near the top of the gel in comparison to the other strains of M. hyopneumoniae. Differences were also found in the relative intensities of the common bands among M. hyopneumoniae strains.

Figure 1. Electrophoretic patterns of mycoplasma cell proteins

(A) M. hyopneumoniae 1361 A
(B) M. hyopneumoniae 1375 C
(C) M. hyopneumoniae 1417
(D) M. hyopneumoniae 1419
(E) M. hyopneumoniae 1424 B
(F) M. hyopneumoniae 1472 C
(G) M. hyopneumoniae 11
(H) M. hyopneumoniae J
(I) M. flocculare Ms 42
(J) M. hyorhinis BTS 7


A
B
C
D
E
G
H
I
J

Two-dimensional immunoelectrophoresis

Antigens from 6 field strains and 4 reference strains of mycoplasmas were compared by means of two-dimensional immunoelectrophoresis using homologous and heterologous antisera. The numbers of precipitin arcs which were distinguished on the electropherograms are listed in Table 4. In homologous reactions, 11-21 precipitin peaks were detected; and 2-20 precipitin peaks were observed in heterologous systems. As shown in Figure 2, 21 components were recognized in strain 1417 by the homologous antiserum. The peaks were labeled by relative migration rates versus bovine albumin which was assigned a value of 1.0. The labeled peaks of 3 different mycoplasma species are shown in Figures 2, 3 and 4; and highly significant differences were detected between the mycoplasma species. Most peaks appeared as symmetrical curves or single peaks, but double peaks also presented on the electropherograms.

Antiserum produced against strain 1361 A of M. hyopneumoniae was strongly reactive with all M. hyopneumoniae strains, and a comparison of these electropherograms is presented in Figure 5. Since the mobilities of peaks were highly reproducible and the standard deviation of the areas was $\pm$ 8% (Thirkill and Kenny, 1975), the relative areas of peaks were determined in 8 runs. Although the peaks 0.40,

Table 4. Comparison of mycoplasma strains by two-dimensional immunoelectrophoresis

Antiserum		Antigen											
		M. hyopneumoniae 11	M. hyopneumoniae 1361 A	M. hyopneumoniae 1375 C	M. hyopneumoniae 1417	M. hyopneumoniae 1419	M. hyopneumoniae 1424 B	M. hyopneumoniae 1472 C	M. hyopneumoniae J	M. flocculare Ms 42	M. hyorhinis BTS 7	Horse serum	Swine serum
M. hyopneumoniae	11	16[a]	11	9	10	10	13	17	8	4	2	2	7
M. hyopneumoniae	1361 A	14	15	14	12	13	13	12	8	6	3	2	5
M. hyopneumoniae	1375 C	15	14	13	10	10	11	13	8	4	3	1	6
M. hyopneumoniae	1417	20	15	18	21	13	11	12	12	5	4	2	4
M. hyopneumoniae	1419	14	11	11	15	13	12	10	12	4	4	2	5
M. hyopneumoniae	1424 B	16	15	12	17	10	14	13	11	6	3	2	5
M. hyopneumoniae	1472 C	14	11	9	10	9	10	17	8	6	3	2	5
M. hyopneumoniae	J	10	9	9	9	11	10	10	11	3	3	2	3
M. flocculare	Ms 42	8	8	6	8	6	4	8	6	14	4	2	4
M. hyorhinis	BTS 7	3	2	2	4	3	4	2	2	3	14	1	3

[a]The numbers represent the number of precipitin lines in two-dimensional immunoelectropherograms.

Figure 2. Two-dimensional immunoelectrophoretic profile of M. hyopneumoniae strain 1417. Ten µl of 1417 antigen were electrophoresed in the first phase (anode to the left of the figure) and developed against 0.5 ml antiserum to strain 1417 in the second phase (anode at the top of the figure). The scale at the bottom of the figure indicates the relative electrophoretic mobilities of the peaks: 1.0 equals the mobility of bovine albumin which peak is identified by the vertical bar at the top of the figure

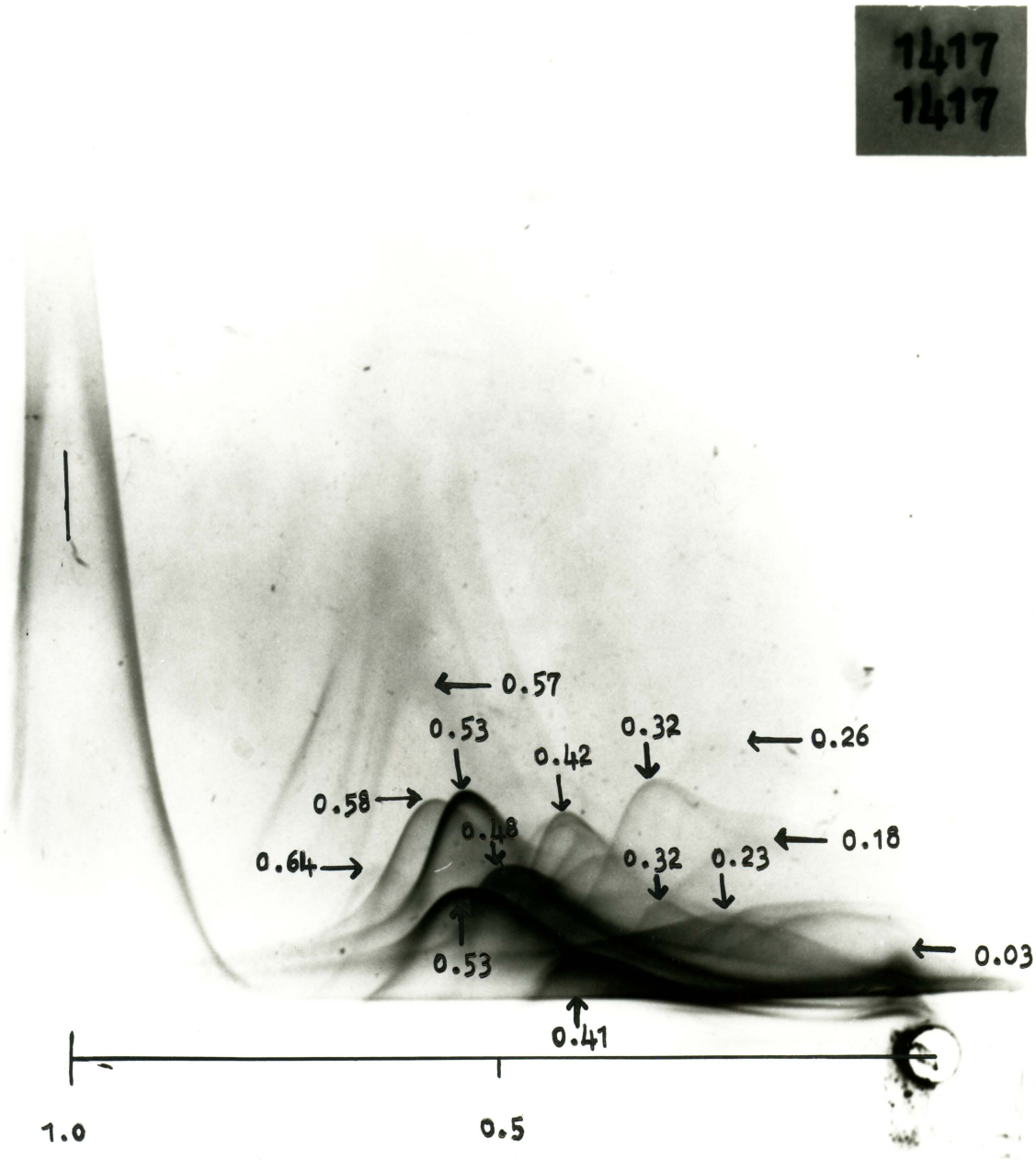

1417
1417
0.57
0.53
0.42
0.32
0.26
0.58
0.48
0.64
0.32
0.23
0.18
0.53
0.03
0.41
1.0
0.5

Figure 3. Two-dimensional immunoelectrophoretic profile of M. flocculare strain Ms 42. Five µl of Ms 42 antigen were electrophoresed in the first phase (anode to the left of the figure) and developed against 0.25 ml antiserum to strain Ms 42 in the second phase (anode at the top of the figure). The scale at the bottom of the figure indicates the relative electrophoretic mobilities of the peaks: 1.0 equals the mobility of bovine albumin which is identified by the vertical bar at the top left of the figure

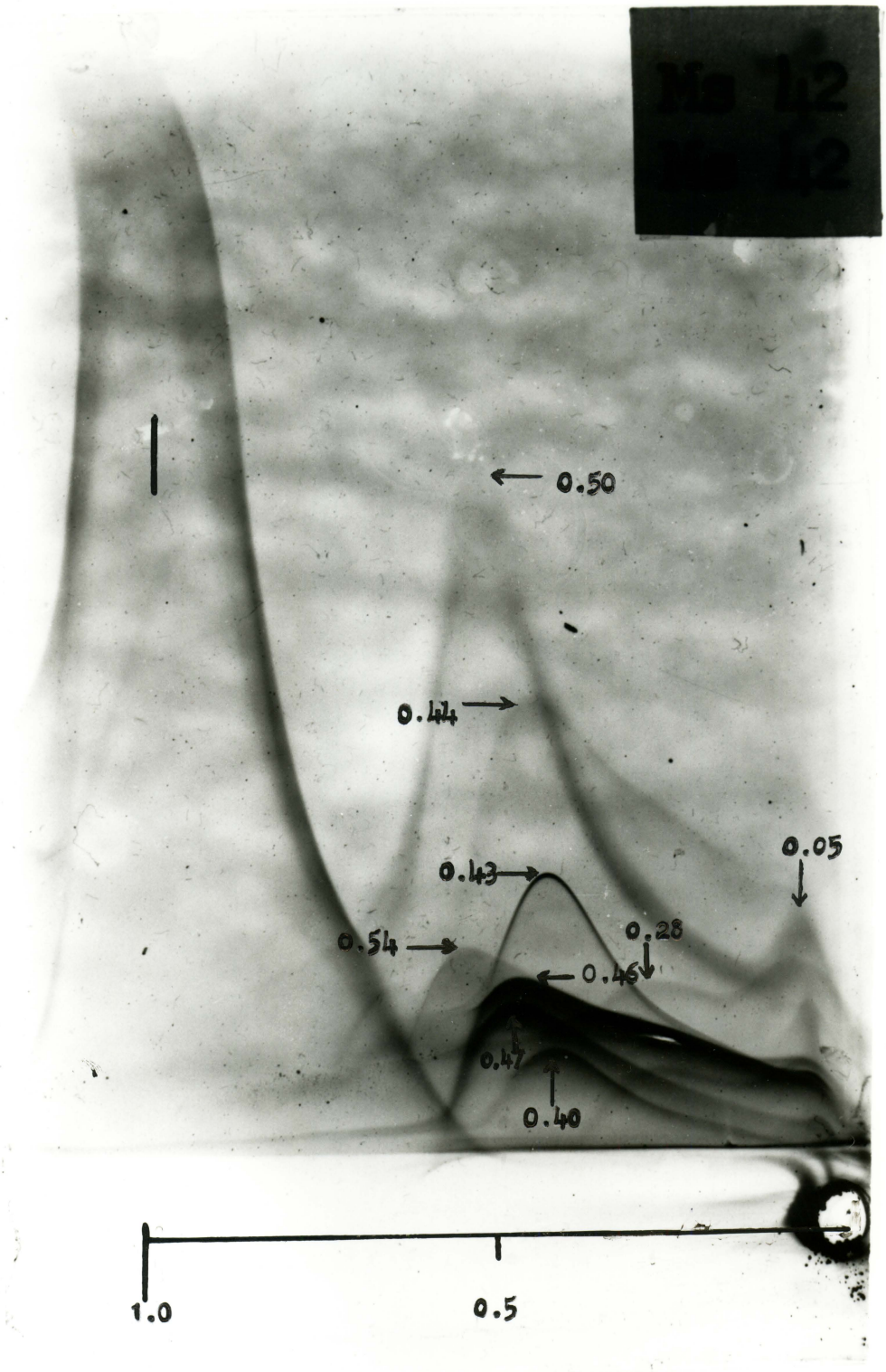

Na 12
12
0.50
0.44
0.43
0.05
0.54
0.28
0.46
0.47
0.40
1.0
0.5

Figure 4. Two-dimensional immunoelectrophoretic profile of M. hyorhinis strain BTS 7. Five µl of BTS 7 antigen were electrophoresed in the first phase (anode to the left of the figure) and developed against 0.25 ml antiserum to strain BTS 7 in the second phase (anode at the top of the figure). The scale at the bottom of the figure indicates the relative electrophoretic mobilities of the peaks: 1.0 equals the mobility of bovine albumin which peak is identified by the vertical bar at the top of the figure

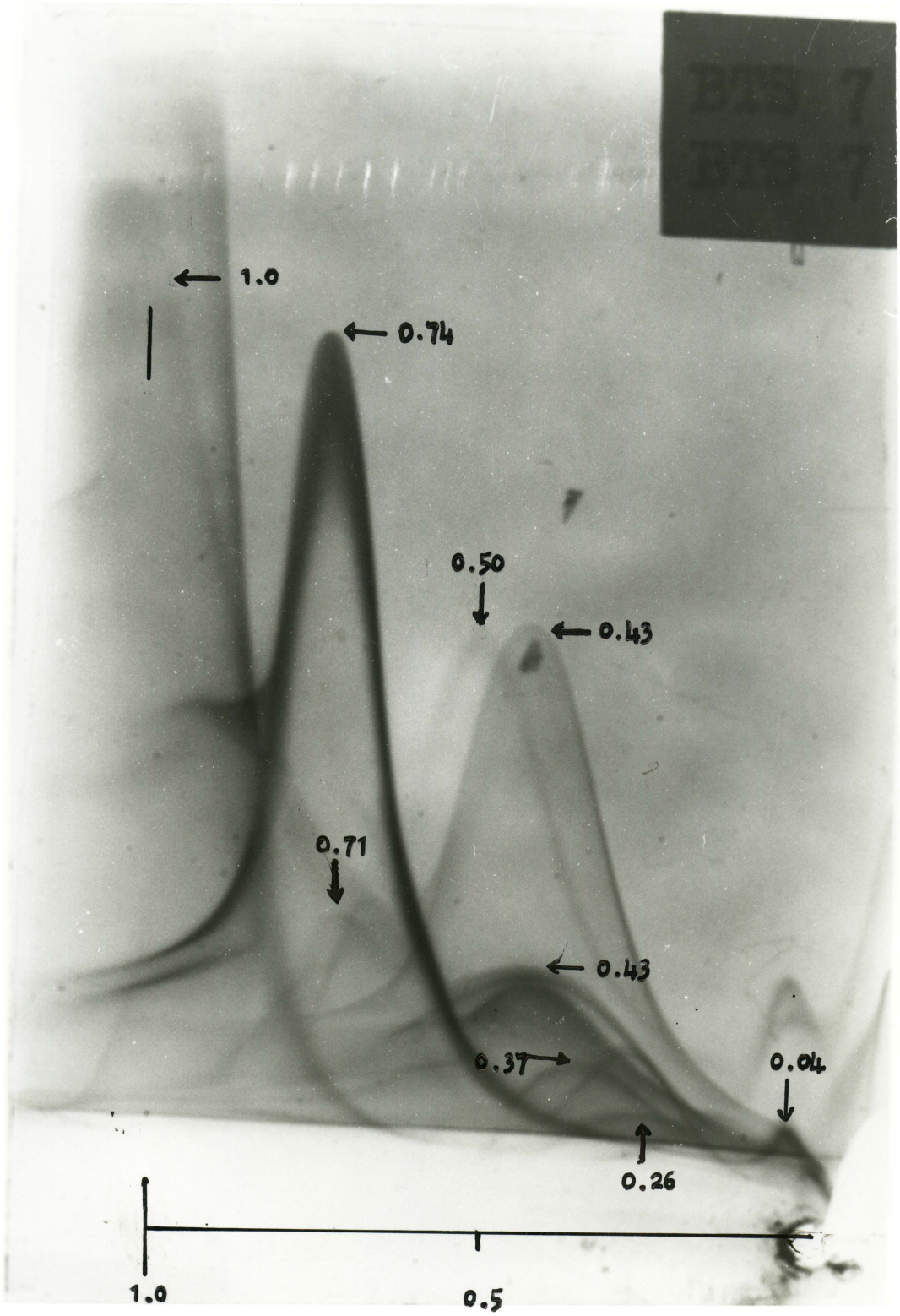
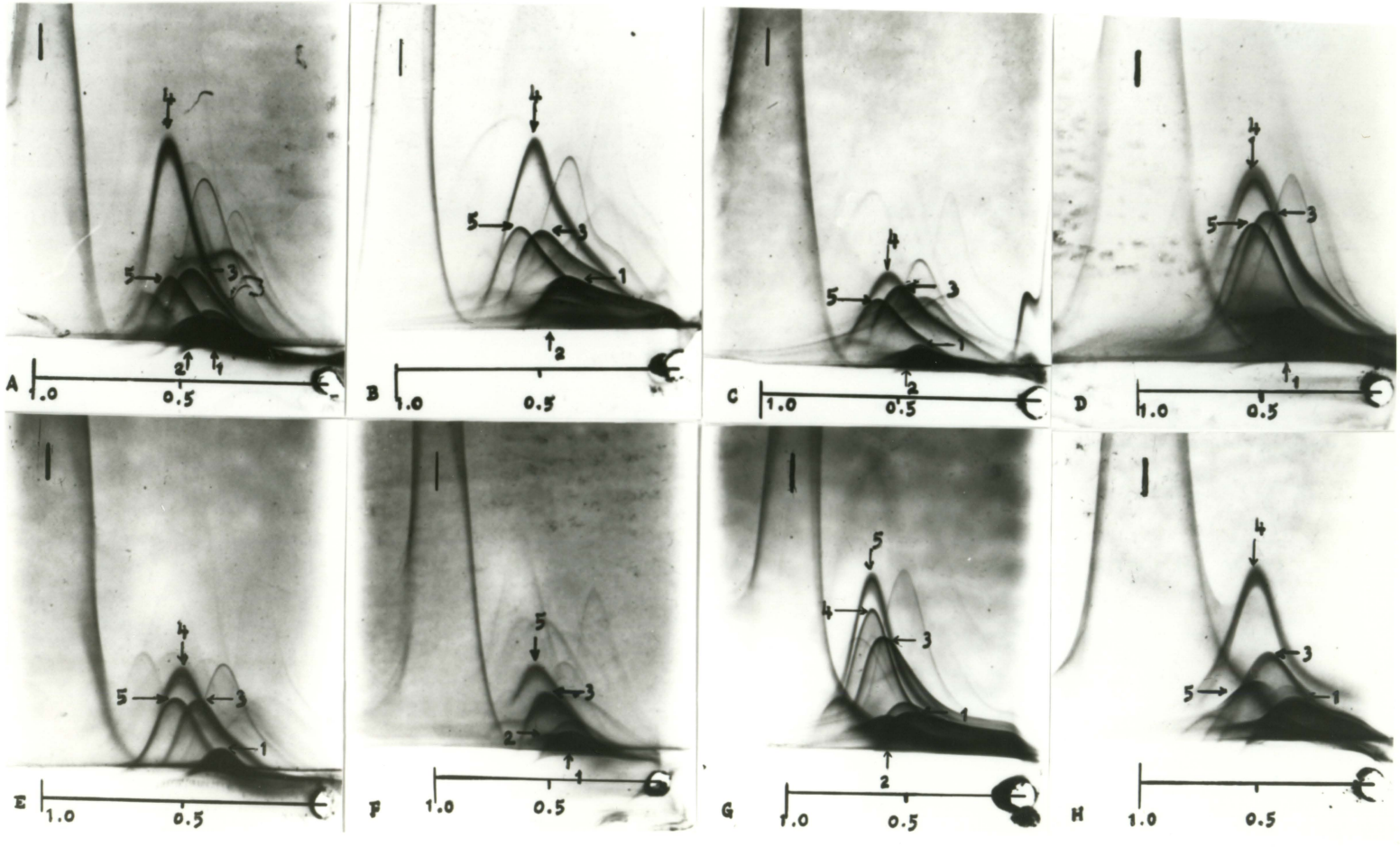

1.0
0.74
0.50
0.43
0.71
0.43
0.37
0.04
0.26
1.0
0.5

Figure 5. Two-dimensional immunoelectrophoretic profiles of M. hyopneumoniae strains against antiserum to M. hyopneumoniae strain 1361 A. Antigens:

(A) M. hyopneumoniae 11
(B) M. hyopneumoniae 1361 A
(C) M. hyopneumoniae 1375 C
(D) M. hyopneumoniae 1417
(E) M. hyopneumoniae 1419
(F) M. hyopneumoniae 1424 B
(G) M. hyopneumoniae 1472 E
(H) M. hyopneumoniae J

(Vertical bar at top left of each frame indicates the center of the bovine albumin control pillar. The relative migration rates of the labeled peaks were determined in 8 runs. Peaks: (1) 0.40, (2) 0.47, (3) 0.50, (4) 0.54, and (5) 0.55)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1.0
0.5

0.47, 0.50, and 0.55 appeared on every profile, the intensity and height of the precipitation lines varied. Peak 0.54 was very intense in strains 11, 1361 A, 1417 and J, but it was lower or fainter on the other profiles. However, dissimilar mobilities and significant antigenic heterogeneities were observed among M. hyopneumoniae strains.

The profiles in Figures 6, 7, 8 and 9 were obtained when strains 11, 1361 A, 1375 C and 1417 were tested against antisera to all mycoplasma strains, respectively, and the profiles were compared by means of the number and intensity of precipitin peaks formed. In Figure 6, antiserum to strain 1375 C produced a profile with strain 11 which showed a few similarities to the profile of strain 1472 C antiserum against strain 11, since some peaks on both electropherograms revealed similar relative mobility and peak height. Although the antisera of M. hyopneumoniae recognized a large number of components in the homologous and heterologous strains, distinct electrophoretic mobility and peak intensity were detected among M. hyopneumoniae strains (Figures 6, 7, 8 and 9).

Antiserum to the Ms 42 strain of M. flocculare recognized 6-8 components in strains 1361 A, 1375 C and 1417 of M. hyopneumoniae, and only 2-4 components in the same strains were recognized by antiserum to BTS 7 strain of

Figure 6. Two-dimensional immunoelectrophoretic profiles of M. hyopneumoniae strain 11 against antisera to M. hyopneumoniae strains. Antisera:

(A) M. hyopneumoniae 11
(B) M. hyopneumoniae 1361 A
(C) M. hyopneumoniae 1375 C
(D) M. hyopneumoniae 1417
(E) M. hyopneumoniae 1419
(F) M. hyopneumoniae 1424 B
(G) M. hyopneumoniae 1472 C
(H) M. hyopneumoniae J

(Vertical bar at top left of each frame indicates the center of the bovine albumin control pillar)

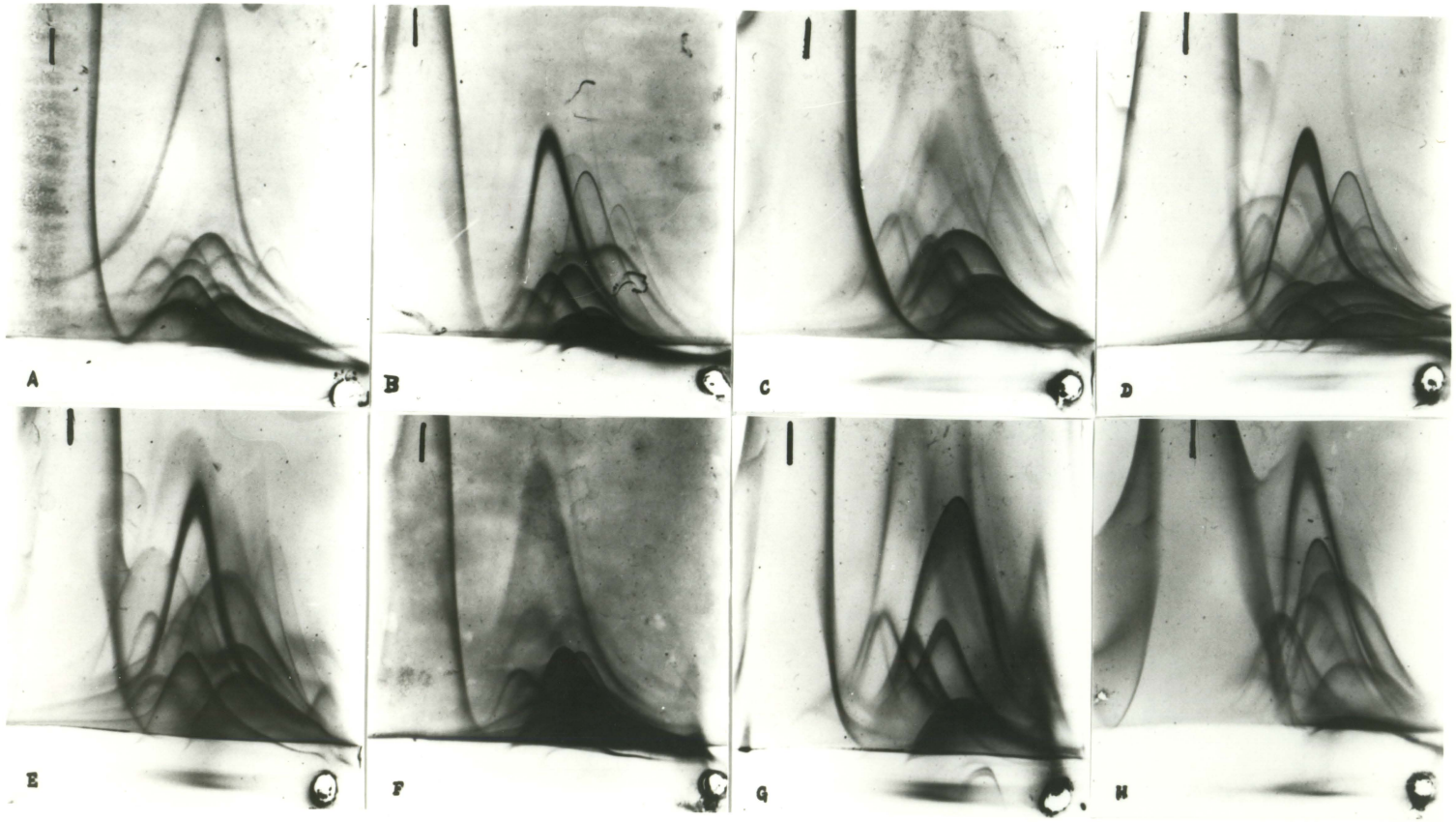
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Figure 7. Two-dimensional immunoelectrophoretic profiles of M. hyopneumoniae strain 1361 A against antisera to mycoplasma strains. Antisera:

(A) M. hyopneumoniae 11
(B) M. hyopneumoniae 1361 A
(C) M. hyopneumoniae 1375 C
(D) M. hyopneumoniae 1417
(E) M. hyopneumoniae 1419
(F) M. hyopneumoniae 1424 B
(G) M. hyopneumoniae 1472 C
(H) M. hyopneumoniae J
(I) M. flocculare Ms 42
(J) M. hyorhinis BTS 7

(Vertical bar at top left of each frame indicates the center of the bovine albumin control pillar)

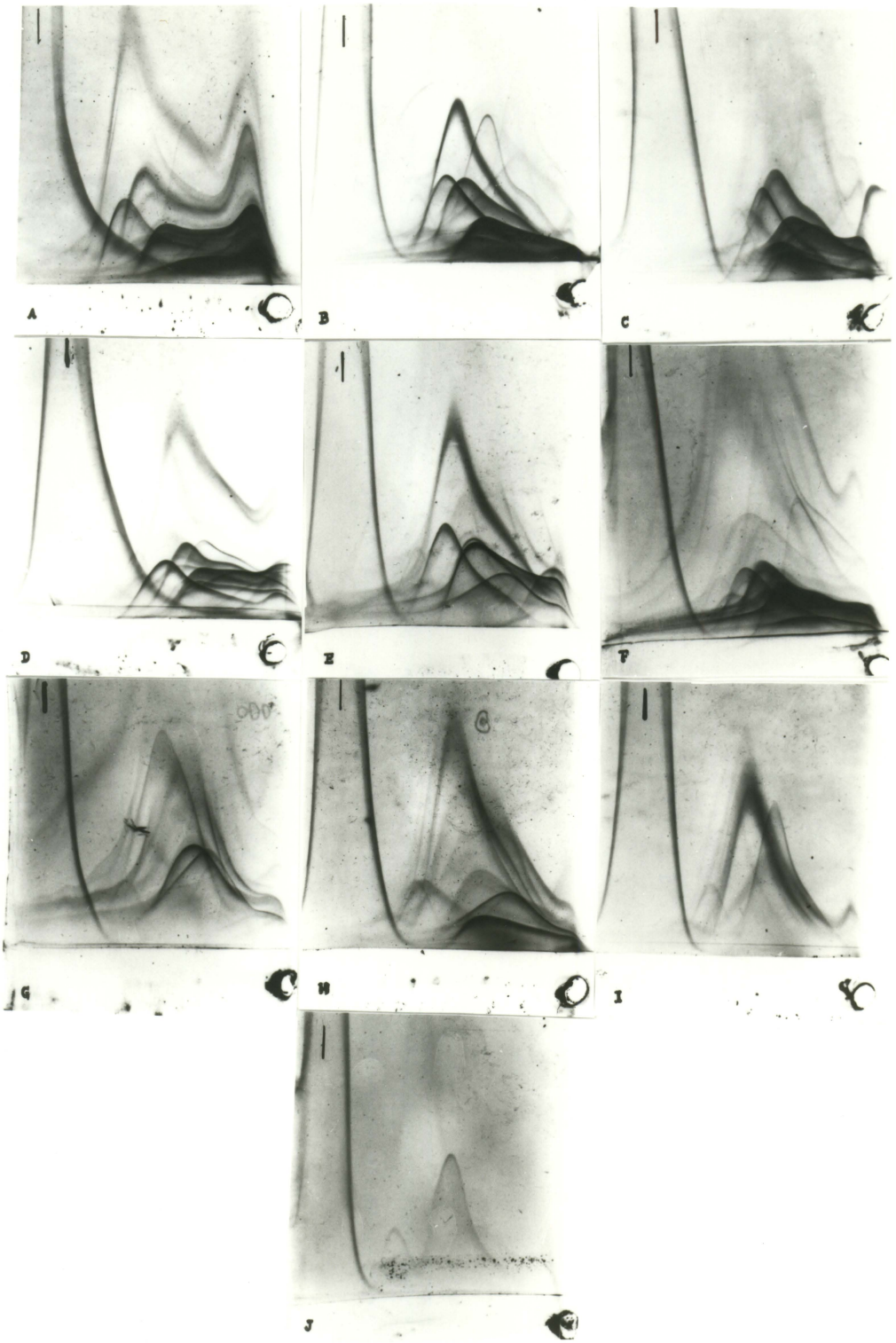
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Figure 8. Two-dimensional immunoelectrophoretic profiles of M. hyopneumoniae strain 1375 C against antisera to mycoplasma strains. Antisera:

(A) M. hyopneumoniae 11
(B) M. hyopneumoniae 1361 A
(C) M. hyopneumoniae 1375 C
(D) M. hyopneumoniae 1417
(E) M. hyopneumoniae 1419
(F) M. hyopneumoniae 1424 B
(G) M. hyopneumoniae 1472 C
(H) M. hyopneumoniae 11
(I) M. flocculare Ms 42
(J) M. hyorhinis BTS 7

(Vertical bar at top left of each frame indicates the center of the bovine albumin control pillar)

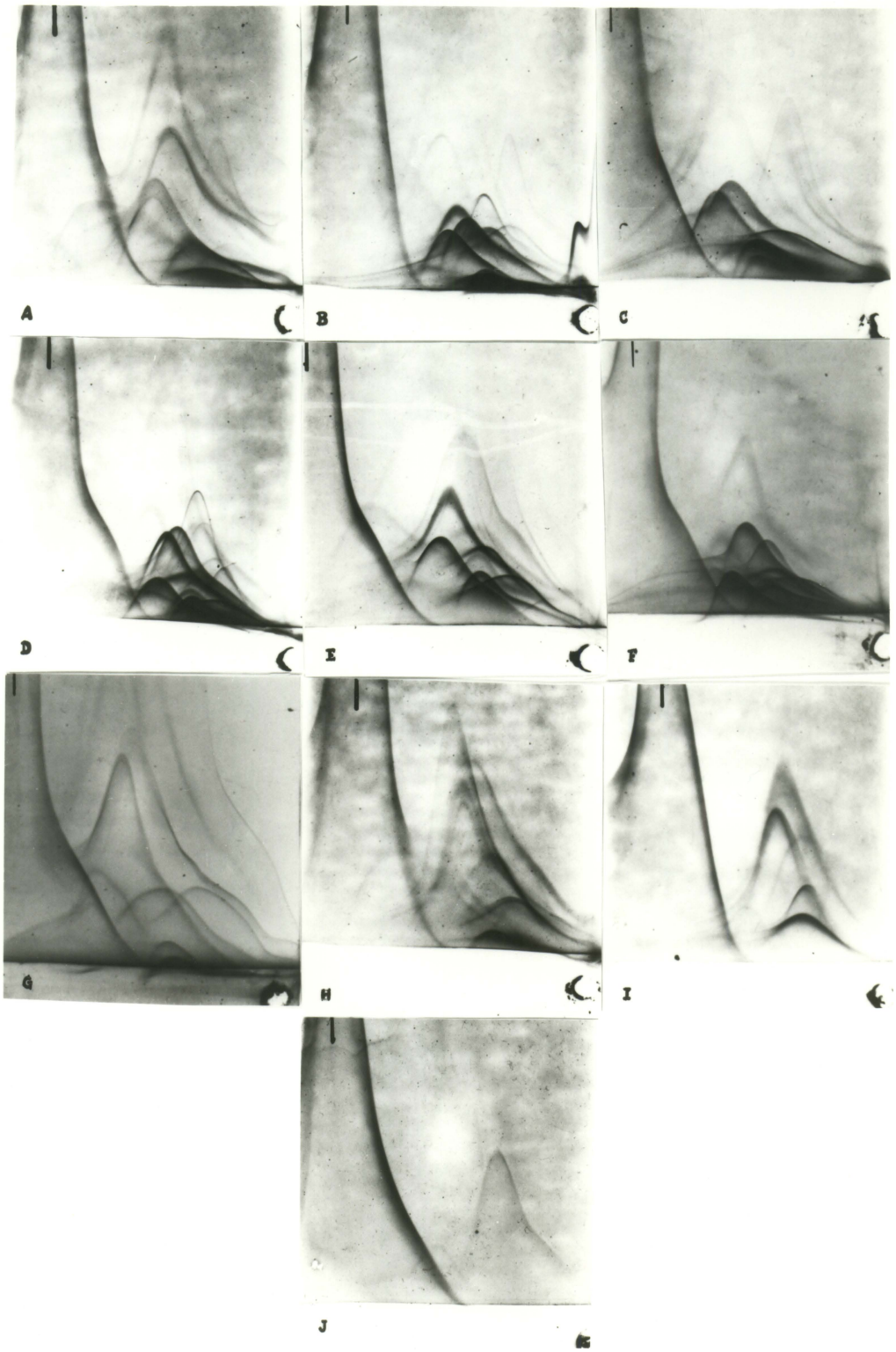
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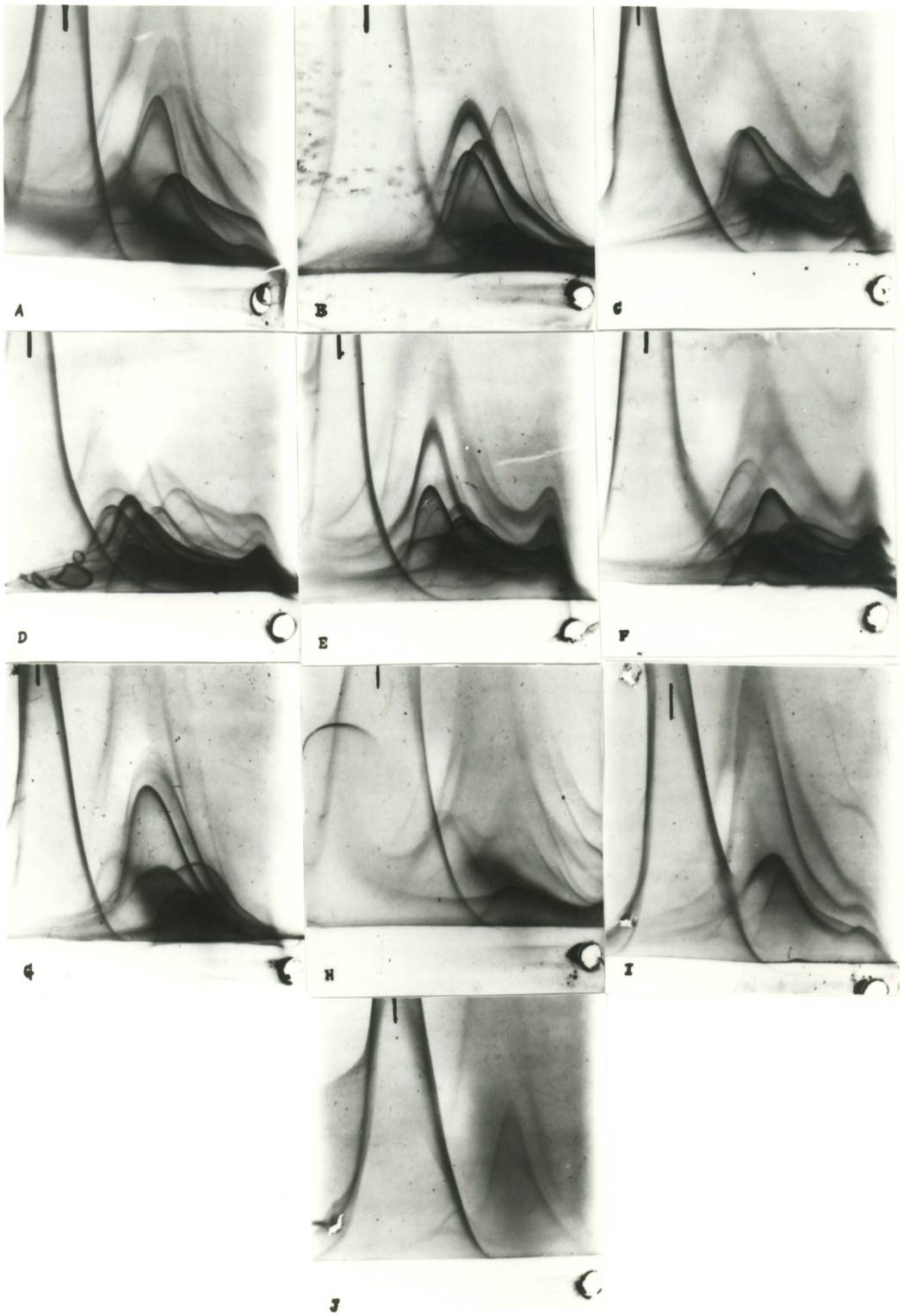
Figure 9. Two-dimensional immunoelectrophoretic profiles of M. hyopneumoniae strain 1417 against antisera to mycoplasma strains. Antisera:

(A) M. hyopneumoniae 11
(B) M. hyopneumoniae 1361 A
(C) M. hyopneumoniae 1375 C
(D) M. hyopneumoniae 1417
(E) M. hyopneumoniae 1419
(F) M. hyopneumoniae 1424 B
(G) M. hyopneumoniae 1472 C
(H) M. hyopneumoniae J
(I) M. flocculare Ms 42
(J) M. hyorhinis BTS 7

(Vertical bar at top left of each frame indicates the center of the bovine albumin control pillar)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M. hyorhinis (Figures 7, 8, and 9). But when the Ms 42 strain and the BTS 7 strain were tested against their homologous antisera, 14 precipitin peaks formed. The large peak, 0.54, in Ms 42 strain (Figure 3) also occurred in each profile obtained when the Ms 42 strain was tested against antisera to M. hyopneumoniae strains. Peak 1.0 and large, strong intensity peak 0.74 obtained with BTS 7 occurred only when it was reacted against its homologous antiserum (Figure 4).

For the controls, each anti-mycoplasmal rabbit serum was tested against horse serum, and 1-2 small and faint peaks with mobilities at 0.1 and 0.73 or at 0.73 only formed. Each antiserum was also tested against swine serum, and 3-7 large peaks were observed.

DISCUSSION

In this study, all mycoplasma strains grew well in Friis broth medium containing 25% swine serum; but the growth of these strains was unsatisfactory in the same basal medium containing 20% horse serum, especially strains 11, 1419 and 1424 B of M. hyopneumoniae. Furthermore, M. hyopneumoniae strains could not be maintained serially in subculture with rabbit muscle infusion broth. The finding that the J strain of M. hyopneumoniae grew better and provided a more satisfactory test antigen for serological procedures than strain 11 of M. hyopneumoniae were in agreement with ICSB Subcommittee on the Taxonomy of Mycoplasmatales (1975) and Rose et al. (1979).

For identification and classification of mycoplasmas by serological methods, production of specific and high-titer antisera is the most basic requirement. The antiserum to strain 1361 A seemed especially satisfactory for identification of M. hyopneumoniae, since this antiserum reacted strongly with every strain of M. hyopneumoniae by means of growth inhibition, metabolic inhibition and two-dimensional immunoelectrophoresis. Friis (1977) reported that antisera produced with mycoplasma immunogen grown in rabbit muscle infusion broth were superior in the growth precipitation test to antisera produced with immunogen grown in Friis broth medium.

Lemcke (1973) also mentioned that the dosage of immunogen, the route and time schedule of immunization were important for the production of hyperimmune antisera against mycoplasmas.

In the growth inhibition test, the field strains of M. hyopneumoniae were shown to be related closely to reference strains 11 and J, although variation of inhibition zone sizes were observed among strains. Differences in zone sizes suggests that there is antigenic heterogeneity among M. hyopneumoniae strains. The distinction of M. flocculare and M. hyorhinis from M. hyopneumoniae was achieved most effectively with this method. As mentioned by other workers, the growth inhibition test was only qualitative and insensitive, but this test possesses the high species-specificity and is suitable for routine identification of mycoplasmas (Purcell et al., 1969; Hollingdale and Lemcke, 1970; Friis, 1974a).

It has been shown that growth inhibiting antibody of mycoplasma develops with membrane preparations (Hollingdale and Lemcke, 1969; Kahane and Razin, 1969). Membranes from M. gallisepticum (Williams and Taylor-Robinson, 1967; Kahane and Razin, 1969; Goel, 1973) and M. hominis (Hollingdale and Lemcke, 1969) elicited production of growth inhibiting and metabolic inhibiting antibodies.

Antigenic relatedness of the field strains and reference strains of M. hyopneumoniae was close, but antigenic diversity was clearly detected within the species with the metabolic inhibition test. The MI titer of antiserum to strain 1361 A was 16-fold higher against strain 1361 A than were titers obtained with antisera to strains 11 and 1419 against the same strain. Conversely the MI titer obtained with homologous antiserum against 1419 was 8-fold lower than when strain 1419 was reacted with antiserum to strain 1361 A. These results suggest that the MI test is more useful for the study of intraspecies antigenic differences than the GI test, since type-specificity was readily apparent with the MI test.

Growth medium containing unheated horse serum was essential for use in the MI test, because the specific inhibitory effect of antisera was greatly decreased when heat-inactivated horse serum was used. Taylor-Robinson et al. (1966) also mentioned that the addition of unheated guinea-pig serum to the MI test did not enhance the MI titer when the MI medium was supplemented with unheated horse serum. Heat inactivation of antisera on the other hand did not reduce the antibody titer but eliminated nonspecific inhibition produced by the pre-inoculation rabbit antiserum (Taylor-Robinson et al., 1966; Fernald et al., 1967).

Membrane antigens involved in complement-mediated immune

killing were distinct among different mycoplasma species (Barker and Patt, 1970; Lin and Kass, 1970; Razin et al., 1970a; Dörner et al., 1976). Metabolic inhibiting antibody to M. pneumoniae was induced by immunization with glycolipids from that organism reaggregated with A. laidlawii membrane. Therefore, it was concluded that glycolipids were the determinants responsible for induction of MI antibody against M. pneumoniae (Razin et al., 1970a, 1971). However, Dörner et al. (1976) reported that membrane proteins participated in the complement-dependent mycoplasmacidal reaction to a greater extent than membrane lipids in A. laidlawii.

Though the electrophoretic patterns of M. hyopneumoniae proteins revealed many similarities, minor variations of bands near the top of the gels and differences in the relative intensities were detected among M. hyopneumoniae strains. Zola et al. (1970) mentioned that minor variations in the relative intensities of bands were observed in different electrophoretic runs and that these were not meaningful. However, those bands in the top one-third of the gel were of greatest value for detection of strain differences (Razin et al., 1970b; Dellinger and Jasper, 1972).

Wreghitt et al. (1974) reported that 3 strains of M. hyosynoviae shared the majority of bands, but differences

in patterns were observed among the strains. Also, intra-species variations in gel patterns obtained with mycoplasma proteins were reported for M. hominis (Razin, 1968), M. pulmonis (Forshaw, 1972) and M. gallisepticum (Rhoades et al., 1974).

In this study, no correlation could be seen between variations in gel patterns and differences detected with the GI and MI tests. The electrophoretic pattern of strain 1424 B lacked 2 weak bands that were seen in strain 1375 C, but the antigenic relatedness of strains 1375 C and 1424 B was close as determined by the MI test.

The gel patterns of M. hyopneumoniae strains were more similar to the Ms 42 strain of M. flocculare than to BTS 7 strain of M. hyorhinis. M. hyorhinis was easily differentiated from M. hyopneumoniae or M. flocculare by polyacrylamide gel electrophoresis. These results were in accordance with Rose et al. (1979) and Nicolet et al. (1980). Although highly complex patterns can not be resolved with one-dimensional polyacrylamide gel electrophoresis, this lower sensitivity makes it very useful for species classification (Razin, 1968).

In two-dimensional immunoelectrophoresis, use of 0.5% Triton X-100 and 0.1% sodium deoxycholate, a neutral detergent and an anionic detergent, in the first phase of electrophoresis provided better resolution in a pre-test

than the use of 0.5% Tween 20 and 0.1% sodium deoxycholate. The method was adapted from Alexander and Kenny (1978) who reported that anodic migration and thus resolution of membrane antigens of M. arginini was greatly improved by incorporating Triton X-100 and sodium deoxycholate in agarose used for electrophoresis in the first dimension. They reported that these detergents had no effect on second phase electrophoresis, therefore, these detergents were omitted from antibody-containing agarose.

Antiserum to strain 1361 A of M. hyopneumoniae was a potent antiserum as evaluated by GI and MI tests. This antiserum was also suitable for two-dimensional immunoelectrophoresis since a large number of components could be demonstrated when it was used. A comparison of M. hyopneumoniae strains tested against antiserum to strain 1361 A is presented in Figure 5. Several common antigenic components were recognized among the strains and antigenic differences were observed also in the profiles. Two-dimensional immunoelectrophoresis provided a useful quantitative analysis of antigens for comparison of M. hyopneumoniae strains. The antigens were characterized by the electrophoretic mobilities relative to bovine albumin. Also, the intensity and height of peaks differed greatly (Figure 5).

When antigen from strain 1361 A was tested against

antisera to all M. hyopneumoniae strains marked differences in electrophoretic mobility and peak intensity were detected, although a few common components were detected (Figure 7). In a comparison of strains of M. arginini, Alexander and Kenny (1977) found that common antigens in strain G-230, leonis and 23243 included membrane and cytoplasmic components. The common components recognized depended upon the ability of the rabbit to recognize them and their relative concentration between strains (Thirkill and Kenny, 1974).

The metabolic inhibition titer obtained when strain 1375 C was tested against its homologous antiserum was 2-fold lower than the titer obtained with antisera to strains 1361 A or 1417 against the same strain (Table 3). As shown in Figure 8, more antigenic components were distinguished by reacting strain 1375 C with antiserum to strain 1361 A or 1417 than by reacting strain 1375 C with the homologous antiserum. Such results observed on the electropherograms were in accordance also with the results detected with the metabolic inhibition test.

With the serological tests, strain 1417 of M. hyopneumoniae was inhibited to a significant degree in the growth inhibition test (9.0 mm) and metabolic inhibition test (>16,384) by the homologous antiserum. By two-dimensional immunoelectrophoresis, strain 1417 formed 21

precipitin peaks in the homologous reaction, when this strain was tested against the heterologous antisera 9-17 precipitin lines formed. Also, marked antigenic heterogeneity was observed among M. hyopneumoniae strains. However, the intra-species antigenic differences shown in the profiles (Figure 9) did not correlate with the antigenic differences detected with the GI (Table 2) and MI (Table 3) tests.

The growth of strains 23243 and leonis of M. arginini was inhibited by the antiserum to strain G-230 in the GI test. Only strain G-230 contained a prominent strain-specific membrane antigen on the cell surface, but these 3 strains of M. arginini did share 1 membrane antigen on the cell surface (Hahn and Kenny, 1974; Alexander and Kenny, 1977).

Major strain specific antigens were demonstrated when the mycoplasma strains were compared by two-dimensional immunoelectrophoresis. Also antigens unique to each strain were detected. If the strain-specific antigens were present in other strains, the concentration of these antigens was minute in those strains (Thirkill and Kenny, 1974, 1975). With M. arginini, growth inhibition and complement-mediated lysis tests were required to detect the strain-specific antigen. The antigen was unable to be judged solely by staining intensity of the precipitin line in an electropherogram (Alexander and Kenny, 1977).

Cross reactions were detected between M. hyopneumoniae and M. flocculare by means of the metabolic inhibition and two-dimensional immunoelectrophoresis tests but not with the growth inhibition test. By two-dimensional immunoelectrophoresis, the Ms 42 strain of M. flocculare showed strong reciprocal cross reactions with M. hyopneumoniae strains. No cross reaction was observed between M. hyorhinis and M. hyopneumoniae or between M. hyorhinis and M. flocculare with the growth inhibition and metabolic inhibition tests. In their study, Meyling and Friis (1972) reported that the Ms 42 strain of M. flocculare was differentiated completely from M. hyopneumoniae and M. hyorhinis with GI and MI tests, but slight cross reactions were detected between M. flocculare and M. hyopneumoniae in agar gel diffusion, complement fixation, immunofluorescence and growth precipitation (Friis, 1977) tests. And the cross reactions noted most likely represented an antigenic relationship between these 2 species. Furthermore, Friis (1974a) also found a slight cross reaction between M. hyorhinis and M. hyopneumoniae in the MI test; and the cross reaction occurred only with strains which were inhibited by their homologous antisera to very high titers.

Although a fast component and some peaks in the BTS 7 strain of M. hyorhinis were only revealed in the homologous reaction, the 3 species did share 1 common component in two-

dimensional immunoelectrophoresis. Antisera produced against M. hyopneumoniae strains reacted more strongly with Ms 42 strain of M. flocculare than with BTS 7 strain of M. hyorhinis. It was apparent that M. flocculare was related more closely to M. hyopneumoniae than M. hyopneumoniae was to M. hyorhinis or M. flocculare to M. hyorhinis. Thirkill and Kenny (1974) reported that common antigens were observed among the non-glycolytic arginine-utilizing mycoplasma species, M. hominis, M. arthritidis, M. gateae, M. gallinarum, and M. arginini, by two-dimensional immunoelectrophoresis. M. arginini showed a strong relationship to M. gateae. But no cross reactions were observed between the nonglycolytic species and a glycolytic species, M. gallisepticum. In this respect, the close relationship of M. flocculare to M. hyopneumoniae is unique among glycolytic mycoplasmas.

The antigens and immunogens used for two-dimensional immunoelectrophoresis were grown in Friis broth medium containing 20% horse serum and rabbit muscle infusion broth, respectively. Since M. hyopneumoniae strains could not be maintained serially in rabbit muscle infusion broth, 60 ml of inoculum grown in Friis broth medium containing 25% swine serum were inoculated in 2 l of rabbit muscle infusion broth for production of the immunogen. It was estimated that the immunogens prepared in this study as described above containing about 0.75% swine serum in rabbit muscle

infusion broth. And the antigens prepared in the manner described contained about 0.000049% swine serum in Friis broth medium containing 20% horse serum; for instance, 0.5 ml of mycoplasma grown in Friis broth medium containing 25% swine serum was subcultured in 5 ml of the same basal medium containing 20% horse serum serially, then 10 ml of the 4th passage was inoculated in 500 ml of Friis broth medium containing 20% horse serum for production of the antigens.

When antiserum to each mycoplasma strain reacted with horse serum, 1-2 faint peaks were observed on the electropherograms. The peaks were also seen in the profile of the same anti-mycoplasmal serum tested against swine serum. In the reactions of swine sera tested against antisera to mycoplasma strains, 3-7 peaks were detected in the profiles since the immunogens were contaminated by swine serum. It has been shown that serum components coprecipitate with the pellet during centrifugation (Yaguzhinskaya, 1976).

In the comparison of arginine-utilizing mycoplasma species by two-dimensional immunoelectrophoresis, the immunogens were grown in fresh yeast dialysate broth (Kenny, 1967) supplemented with 10% agamma rabbit serum, and the antigens were grown in "hot" yeast dialysate, soy peptone broth (Pollock and Bonner, 1969) incorporated with 10% agamma horse serum. The medium components did not influence the specificity of this technique (Thirkill and Kenny, 1974). Although

antigenic analysis of mycoplasma was not influenced by medium components, chemical analysis of the antigens could be erroneous since the pellets involved did contain medium components (Kenny, 1979).

Nicolet et al. (1980) reported that a glycoprotein of swine serum contaminated mycoplasma cultures, and the glycoprotein confused serological cross reactions when the contaminated mycoplasma antigens reacted with rabbit antisera. They also observed that cross reactions occurred between antiserum to swine serum precipitates and M. hyopneumoniae grown in media containing swine serum and M. hyorhinis grown in media containing horse serum by means of double-diffusion test. In the former reaction 2 precipitin lines formed, and 1 precipitin line was observed in the latter reaction.

In our study, greater antigenic heterogeneity was observed among M. hyopneumoniae strains by two-dimensional immunoelectrophoresis than by means of the other serological tests and polyacrylamide gel electrophoresis. Since the growth inhibiting and metabolic inhibiting antibodies very likely reacted with membrane antigens on the cell surface as has been shown with other mycoplasmas; in Coomassie-stained electrophoretic patterns, mycoplasma cell proteins could be detected (Razin and Rottem, 1967); and in Coomassie-

stained two-dimensional immunoelectropherograms, membrane protein antigens and a soluble fraction of cytoplasmic antigens could be quantitated as reported by Alexander and Kenny (1977, 1978).

Although the strain 11 of M. hyopneumoniae and J strain of M. hyopneumoniae possessed a strong relationship revealed by growth inhibition, metabolic inhibition and polyacrylamide gel electrophoresis methods, significant antigenic differences between strains 11 and J were observed by two-dimensional immunoelectrophoresis. Hollingdale and Lemcke (1970) found that antigens responsible for strain specificity among strains of M. hominis were in the membrane but not in a soluble fraction. Alexander and Kenny (1977) made similar observations with M. arginini.

SUMMARY

Six field strains of M. hyopneumoniae isolated from pneumonic lung of pigs and 4 mycoplasma reference strains were compared by means of serologic and electrophoretic techniques. Using the growth inhibition test, antigenic heterogeneity was observed among the strains of M. hyopneumoniae, although they were related closely. No cross reactions were detected between M. hyopneumoniae, M. flocculare and M. hyorhinis with the growth inhibition test. The metabolic inhibition test was more useful for comparison of intra-species antigenic difference than the growth inhibition test, since antigenic diversity was clearly detected among M. hyopneumoniae strains. Although cross reactions occurred between M. hyopneumoniae and M. hyorhinis or between M. flocculare and M. hyorhinis in the metabolic inhibition test, slight cross reactions were observed between M. hyopneumoniae and M. flocculare. Using polyacrylamide gel electrophoresis, minor variations were detected in patterns of M. hyopneumoniae proteins; however, the majority of bands were shared. Clear differences were seen between patterns obtained with the 3 different mycoplasma species. By two-dimensional immunoelectrophoresis, significant antigenic differences were observed among M. hyopneumoniae strains, although some common components revealed on electropherograms.

M. flocculare possessed an antigenic relationship to M. hyopneumoniae as determined by two-dimensional immunoelectrophoresis; whereas marked differences were observed between M. hyopneumoniae and M. hyorhinis and between M. flocculare and M. hyorhinis. Evidence obtained in this study indicates strains of mycoplasma tentatively identified as M. hyopneumoniae were closely related; however, evidence was obtained also of minor diversity in antigenic makeup among these strains.

BIBLIOGRAPHY

Ackroyd, P. 1967. Acrylamide gel slab electrophoresis in a simple glass cell for improved resolution and comparison of serum proteins. Anal. Biochem. 19:399-410.

Al-Aubaidi, J. M., A. H. Dardiri and J. Fabricant. 1972. Biochemical characterization and antigenic relationship of Mycoplasma mycoides subsp. mycoides Freundt and Mycoplasma mycoides subsp. capri (Edward) Freundt. Int. J. Syst. Bacteriol. 22:155-164.

Alexander, A. G. and G. E. Kenny. 1977. Characterization of membrane and cytoplasmic antigens of Mycoplasma arginini by two-dimensional (crossed) immunoelectrophoresis. Infect. Immun. 15:313-321.

Alexander, A. G. and G. E. Kenny. 1978. Application of charge shift electrophoresis to antigenic analysis of mycoplasmic membranes by two-dimensional (crossed) immunoelectrophoresis. Infect. Immun. 20:861-863.

Amanfu, W. 1980. Diagnosis of mycoplasmal pneumonia of swine and control of the disease by farrowing seronegative sows. Unpublished M.S. thesis. Library, Iowa State University, Ames, Ia.

Archer, D. B. 1979. Immunoprecipitation of Triton X-100-solubilized Mycoplasma mycoides proteins. J. Gen. Microbiol. 115:111-116.

Armstrong, C. H. 1976. A diagnostically practical approach to isolating and identifying mycoplasmas of porcine origin. Proc. Am. Assoc. Vet. Lab. Diag. 75-91.

Armstrong, D. and B. Yu. 1970. Characterization of canine mycoplasmas by polyacrylamide gel electrophoresis and immunodiffusion. J. Bacteriol. 104:295-299.

Barker, L. F. and J. K. Patt. 1970. Role of complement in immune inactivation of Mycoplasma gallisepticum. J. Bacteriol. 94:403-408.

Betts, A. O. 1952. Respiratory diseases of pigs. V. Some clinical and epidemiological aspects of virus pneumonia of pigs. Vet. Rec. 64:283-288.

Betts, A. O. and P. Whittlestone. 1963. Enzootic or virus pneumonia of pigs: Production of pneumonia with tissue culture fluids. Res. Vet. Sci. 4:471-479.

Beveridge, W. I. B. and A. O. Betts. 1953. Virus pneumonia of pigs. Int. Vet. Congr., Stockholm. Proc. 15, Part 1: 345-349.

Boden, K. and H. Kirchhoff. 1977. Characterization of Acholeplasma strains by horizontal polyacrylamide flat gel electrophoresis. Zentralbl. Bakteriol. Hyg. I Abt. Orig. A 237:342-350.

Braude, R. and S. Plonka. 1975. Effect of enzootic pneumonia on the performance of growing pigs. Vet. Rec. 96: 359-360.

Clyde, W. A. 1964. Mycoplasma species identification based upon growth inhibition by specific antisera. J. Immunol. 92:958-965.

Cole, B. C., C. E. Graham and J. R. Ward. 1970. The isolation of mycoplasmas from chimpanzees. Pp. 390-409 in G. H. Bourne, ed. The Chimpanzee, Vol. II. S. Karger, Basel and New York.

Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121:404-427.

Deeb, B. J. and G. E. Kenny. 1967. Characterization of Mycoplasma pulmonis variants from rabbits. II. Basis for differentiation of antigenic subtypes. J. Bacteriol. 93: 1425-1429.

Del Giudice, R. A., N. F. Robillard and T. R. Carski. 1967. Immunofluorescence identification of mycoplasma on agar by use of incident illumination. J. Bacteriol. 93:1205-1209.

Del Giudice, R. A., T. R. Carski, M. F. Barile, R. M. Lemcke and J. G. Tully. 1971. Proposal for classifying human strain navel and related simian mycoplasmas as Mycoplasma primatum sp. n. J. Bacteriol. 108:439-445.

Dellinger, J. D. and D. E. Jasper. 1972. Polyacrylamide-gel electrophoresis of cell proteins of mycoplasmas isolated from cattle and horses. Am. J. Vet. Res. 33:769-775.

Dinter, Z. and D. Taylor-Robinson. 1969. Susceptibility and resistance of various strains of Mycoplasma hyorhinis to antisera, polymyxins and low pH values. J. Gen. Microbiol. 57:263-272.

Dörner, I., H. Brunner, H. -G. Schiefer and H. -J. Wellensiek. 1976. Complement-mediated killing of Acholeplasma laidlawii by antibodies to various membrane components. Infect. Immun. 13:1663-1670.

Ernø, H. and K. Jurmanová. 1973. Bovine mycoplasmas: Serological studies by double immunodiffusion, growth precipitation and growth inhibition. Acta Vet. Scand. 14: 524-537.

Ernø, H. and M. M. Salih. 1980. The growth precipitation test as a diagnostic method for differentiation of Mycoplasma and Acholeplasma species. Acta Vet. Scand. 21:469-481.

Etheridge, J. R., G. S. Cottew and L. C. Lloyd. 1979. Isolation of Mycoplasma hyopneumoniae from lesions in experimentally infected pigs. Aust. Vet. J. 55:356-359.

Fernald, G. W., W. A. Clyde and F. W. Kenny. 1967. Factors influencing growth inhibition of Mycoplasma pneumoniae by immune sera. Proc. Soc. Exp. Biol. Med. 126:161-166.

Forshaw, K. A. 1972. Electrophoretic patterns of strains of Mycoplasma pulmonis. J. Gen. Microbiol. 72:493-499.

Fowler, R. C., D. W. Coble, N. C. Kramer and T. M. Brown. 1963. Starch gel electrophoresis of a fraction of certain of the pleuropneumonia-like group of microorganisms. J. Bacteriol. 86:1145-1151.

Friis, N. F. 1969. Mycoplasma suipneumoniae isolated in Denmark. Acta Vet. Scand. 10:295-297.

Friis, N. F. 1970. A new porcine Mycoplasma species. Mycoplasma suidaniae. Acta Vet. Scand. 11:487-490.

Friis, N. F. 1971a. Mycoplasmas cultivated from the respiratory tract of Danish pigs. Acta Vet. Scand. 12:69-79.

Friis, N. F. 1971b. Sensitivity of Mycoplasma suipneumoniae to penicillin-G. Acta Vet. Scand. 12:120-121.

Friis, N. F. 1971c. A selective medium for Mycoplasma suipneumoniae. Acta Vet. Scand. 12:454-456.

Friis, N. F. 1973. Resistance of porcine mycoplasmas to drying. Acta Vet. Scand. 14:489-491.

Friis, N. F. 1974a. Mycoplasmas in pigs. Ph.D. dissertation. Royal Vet. Agric. Univ., Copenhagen, Denmark.

Friis, N. F. 1974b. Resistance of porcine mycoplasmas to heat. Acta Vet. Scand. 15:283-285.

Friis, N. F. 1975. Some recommendations concerning primary isolation of Mycoplasma suipneumoniae and Mycoplasma flocculare: A survey. Nord. Vet. Med. 27:337-339.

Friis, N. F. 1976. A serological variant of Mycoplasma hyorhinis recovered from the conjunctiva of swine. Acta Vet. Scand. 17:343-353.

Friis, N. F. 1977. Mycoplasma suipneumoniae and Mycoplasma flocculare in the growth precipitation test. Acta Vet. Scand. 18:168-175.

Friis, N. F. 1979. Selective isolation of slowly growing acidifying mycoplasmas from swine and cattle. Acta Vet. Scand. 20:607-609.

Furlong, S. L. and A. J. Turner. 1975. Isolation of Mycoplasma hyopneumoniae and its association with pneumonia of pigs in Australia. Aust. Vet. J. 51:28-31.

Goel, M. C. 1973. New method of the isolation of membranes from Mycoplasma gallisepticum. J. Bacteriol. 116:994-1000.

Gois̆, M. and F. Kuksa. 1975. Diagnosis and differentiation of porcine mycoplasmas by the growth precipitation test. Zentralbl. Vet. Med. B 22:850-855.

Goiš, M. and D. Taylor-Robinson. 1972. The identification and characterization of some porcine arginine-utilizing mycoplasma strains. J. Med. Microbiol. 5:47-54.

Goiš, M., F. Kuksa, J. Franz and D. Taylor-Robinson. 1974. The antigenic differentiation of seven strains of Mycoplasma hyorhinis by growth inhibition, metabolic inhibition, latex-agglutination, and polyacrylamide-gel-electrophoresis tests. J. Med. Microbiol. 7:105-115.

Golightly-Rowland, L., B. C. Cole, J. R. Ward and B. B. Wiley. 1970. Effect of animal passage on arthritogenic and biological properties of Mycoplasma arthritidis. Infect. Immun. 1:538-545.

Goodwin, R. F. W. 1972. The survival of Mycoplasma suipneumoniae in liquid medium, on solid medium and in pneumonic tissue. Res. Vet. Sci. 13:203-204.

Goodwin, R. F. W. 1976. An improved medium for the isolation of Mycoplasma suipneumoniae. Vet. Rec. 98:260-261.

Goodwin, R. F. W. and J. M. W. Hurrell. 1970. Further observations on the problem of isolating Mycoplasma suipneumoniae from field cases of enzootic pneumonia in pigs. J. Hyg. (Camb.) 68:313-325.

Goodwin, R. F. W. and J. E. Pryor. 1970. Direct isolation of Mycoplasma suipneumoniae on solid medium from pneumonic lesions in pigs. Vet. Rec. 87:726-727.

Goodwin, R. F. W. and P. Whittlestone. 1963. Production of enzootic pneumonia in pigs with an agent grown in tissue culture from the natural disease. Br. J. Exp. Pathol. 44:291-299.

Goodwin, R. F. W. and P. Whittlestone. 1964. Production of enzootic pneumonia in pigs with a microorganism grown in medium free from living cells. Vet. Rec. 76:611-613.

Goodwin, R. F. W. and P. Whittlestone. 1966. Enzootic pneumonia of pigs: Growth and behavior of the causal mycoplasma in liquid media. Br. J. Exp. Pathol. 47:518-524.

Goodwin, R. F. W., A. P. Pomeroy and P. Whittlestone. 1965. Production of enzootic pneumonia in pigs with mycoplasma. Vet. Rec. 77:1247-1249.

Goodwin, R. F. W., A. P. Pomeroy and P. Whittlestone. 1967. Characterization of M. suipneumoniae. A mycoplasma causing enzootic pneumonia of pigs. J. Hyg. (Camb.) 65:85-96.

Goodwin, R. F. W., A. P. Pomeroy and P. Whittlestone. 1968. Attempts to recover Mycoplasma suipneumoniae from experimental and natural cases of enzootic pneumonia in pigs. J. Hyg. (Camb.) 66:595-603.

Gulrajani, T. S. and W. I. B. Beveridge. 1951. Studies on respiratory diseases of pigs. IV. Transmission of infectious pneumonia and its differentiation from swine influenza. J. Comp. Pathol. Ther. 61:118-139.

Hahn, R. G. and G. E. Kenny. 1974. Differences in arginine requirement for growth among arginine-utilizing mycoplasma species. J. Bacteriol. 117:611-618.

Hansen, E. J., R. M. Wilson and J. B. Baseman. 1979a. Isolation of mutants of Mycoplasma pneumoniae defective in hemadsorption. Infect. Immun. 23:903-906.

Hansen, E. J., R. M. Wilson and J. B. Baseman. 1979b. Two-dimensional gel electrophoresis comparison of proteins from virulent and avirulent strains of Mycoplasma pneumoniae. Infect. Immun. 24:468-475.

Hansen, E. J., R. M. Wilson, W. A. Clyde and J. B. Baseman. 1981. Characterization of hemadsorption-negative mutants of Mycoplasma pneumoniae. Infect. Immun. 32:127-136.

Hayflick, L. and W. R. Stinebring. 1955. Intracellular growth of pleuropneumonia-like organisms. Anat. Rec. 121: 477-478.

Heitmann, J. and H. Kirchhoff. 1978. Acholeplasma species differentiation with the growth precipitation test. Acta Vet. Scand. 18:168-175.

Helenius, A. and K. Simons. 1977. Charge shift electrophoresis: Simple method for distinguishing between amphiphilic and hydrophilic proteins in detergent solution. Proc. Natl. Acad. Sci. U.S.A. 74:529-532.

Herderscheê, D. 1963. An improved medium for the cultivation of the Eaton agent. Antonie van Leeuwenhoek. J. Microbiol. Serol. 29:154-156.

Hjärre, A., Z. Dinter and K. Bakos. 1952. Vergleichende Untersuchungen über eine Influenzaähnliche Schweinekrankheit in Schweden und Shopes Schweine influenza. Nord. Vet. 4: 1025-1045.

Hodges, R. T., A. O. Betts and A. R. Jennings. 1969. Production of pneumonia in gnotobiotic pigs with pure cultures of M. hyopneumoniae. Vet. Rec. 84:268-273.

Hollingdale, M. R. and R. M. Lemcke. 1969. The antigens of Mycoplasma hominis. J. Hyg. (Camb.) 67:585-602.

Hollingdale, M. R. and R. M. Lemcke. 1970. Antigenic differences within the species Mycoplasma hominis. J. Hyg. (Camb.) 68:469-477.

Huhn, R. G. 1969. Chemoprophylaxis, chemotherapy, and economic effect of swine enzootic pneumonia. Unpublished Ph.D. dissertation. Library, Iowa State University, Ames, Ia.

Huijsmans-Evers, A. G. M. and A. C. Ruys. 1956. Microorganisms of the pleuropneumonia group (family of Mycoplasmataceae) in man. II. Serological identification and discussion of pathogenicity. Antonie van Leeuwenhoek. J. Microbiol. Serol. 22:377-384.

Jansson, R. 1974. Evaluation of some serological techniques for the identification of Mycoplasma hyorhinis and Mycoplasma suipneumoniae. Acta Vet. Scand. 15:274-282.

Jensen, K. E. 1964. Antibodies to Mycoplasma pneumoniae measured by inhibition of tetrazolium reduction. Bacteriol. Proc. 70-71.

Jensen, P. T., C. Wolstrup and N. F. Friis. 1978. Utilisation of glucose by Mycoplasma suipneumoniae and Mycoplasma flocculare. Acta Vet. Scand. 19:170-183.

Johansson, K. and S. Hjertén. 1974. Localization of the Tween 20-soluble membrane proteins of Acholeplasma laidlawii by cross immunoelectrophoresis. J. Mol. Biol. 86:341-348.

Kahane, I. and S. Razin. 1969. Immunological analysis of mycoplasma membranes. J. Bacteriol. 100:187-194.

Kenny, G. E. 1967. Heat lability and organic solvent-solubility of mycoplasma antigens. Ann. N.Y. Acad. Sci. 143:676-681.

Kenny, G. E. 1979. Antigenic determinants. Pp. 351-380 in M. F. Barile and S. Razin, eds. The Mycoplasma. Vol. I. Academic Press, New York.

Köbe, K. 1933. Die Aetiologie der Ferkelgrippe (enzootische Pneumonie des Ferkels). Zentralbl. Bakteriol. Parasitkde, I Orig,129:161-176.

Krogsgaard-Jensen, A. 1972. Mycoplasma: growth precipitation as a serodiagnostic method. Applied Microbiol. 23: 553-558.

Lamont, H. G. 1938. The problems of the practitioner in connection with the differential diagnosis and treatment of the disease of young pigs. Vet. Rec. 50:1377-1400.

Lannek, N. and T. Wesslén. 1957. Evidence that the SEP agent is an etiological factor in enzootic pneumonia in swine. Nord. Vet. 9:177-190.

Laurell, C. B. 1965. Antigen-antibody crossed electrophoresis. Anal. Biochem. 10:358-361.

Leach, R. H. 1976. The inhibitory effect of arginine on growth of some mycoplasmas. J. Applied Bacteriol. 41:259-264.

Leach, R. H. and M. Butler. 1966. Comparisons of mycoplasmas associated with human tumors, leukemia and tissue cultures. J. Bacteriol. 91:934-941.

L'Ecuyer, C. 1962. Propagation of the primary agent of virus pneumonia of pigs and determination of the microbiology of naturally occurring cases. Unpublished Ph.D. dissertation. Library, Iowa State University, Ames, Ia.

L'Ecuyer, C. 1969. Enzootic pneumonia in pigs: Propagation of a causative mycoplasma in cell cultures and in artificial medium. Can. J. Comp. Med. Vet. Sci. 33:10-19.

L'Ecuyer, C. and P. Boulanger. 1970. Enzootic pneumonia of pigs. Identification of causative mycoplasma in infected pigs and in cell cultures by immunofluorescent staining. Can. J. Comp. Med. Vet. Sci. 34:38-46.

L'Ecuyer, C. and W. P. Switzer. 1963. Virus pneumonia of pigs: Attempts at propagation of causative agent in cell cultures and chicken embryo. Can. J. Comp. Med. Vet. Sci. 27:91-99.

Lee, G. Y. and G. E. Kenny. 1980. Electrophoretic and immunologic heterogeneity of the superoxide dismutases in the Acholeplasmataceae. P. 39 in Abstracts, Third Conference of the International Organization for Mycoplasmology, Custer, South Dakota, Sept. 3-9.

Lemcke, R. M. 1964. The serological differentiation of mycoplasma strains (pleuropneumonia-like organisms) from various sources. J. Hyg. (Camb.) 62:199-219.

Lemcke, R. M. 1973. Serological reactions of mycoplasmas. Ann. N.Y. Acad. Sci. 225:46-53.

Lin, J. S. and E. H. Kass. 1970. Immune inactivation of T-strain Mycoplasmas. J. Infect. Dis. 122:93-95.

Lipman, R. P., W. A. Clyde and F. W. Denny. 1969. Characteristics of virulent, attenuated, and avirulent Mycoplasma pneumoniae strains. J. Bacteriol. 100:1037-1043.

Liu, C. 1957. Studies on primary atypical pneumonia. I. Localization, isolation and cultivation of a virus in chicken embryos. J. Exp. Med. 106:455-467.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement and the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Maré, C. J. 1965. Characterization of a causative agent of virus pneumonia of pigs. Unpublished Ph.D. dissertation. Library, Iowa State University, Ames, Ia.

Maré, C. J. and W. P. Switzer. 1965. New species: Mycoplasma hyopneumoniae a causative agent of virus pneumonia of pigs. Vet. Med. 60:841-846.

Maré, C. J. and W. P. Switzer. 1966a. Virus pneumonia of pigs. Filtration and visualization of a causative agent. Am. J. Vet. Res. 27:1677-1686.

Maré, C. J. and W. P. Switzer. 1966b. Virus pneumonia of pigs. Propagation and characterization of a causative agent. Am. J. Vet. Res. 27:1687-1693.

Marley, J., P. B. Spradbrow and D. A. Watt. 1971. The isolation of mycoplasmas from porcine pneumoniae. Aust. Vet. J. 47:375-378.

Mebus, C. A. and N. R. Underdahl. 1977. Scanning electron microscopy of trachea and bronchi from gnotobiotic pigs inoculated with Mycoplasma hyopneumoniae. Am. J. Vet. Res. 38:1249-1254.

Meyling, A. and N. F. Friis. 1972. Serological identification of a new porcine mycoplasma species, M. flocculare. Acta Vet. Scand. 13:287-289.

Nicol, C. S. and D. G. ff. Edward. 1953. Role of organisms of the pleuropneumonia group in human genital infections. Br. J. Vener. Dis. 29:141-150.

Nicolet, J., PH. Paroz and B. Kristensen. 1980. Growth medium constituents contaminating mycoplasma preparations and their role in the study of membrane glycoproteins in porcine mycoplasmas. J. Gen. Microbiol. 119:17-26.

Paroz, PH. and J. Nicolet. 1978. Glycoproteins in mycoplasmas. Contamination with serum aggregates from growth media. Experientia 34:1668.

Piffer, I. A. 1981. Effect of age on the susceptibility of pigs to Mycoplasma hyopneumoniae pneumonia. Unpublished M.S. thesis. Library, Iowa State University, Ames, Ia.

Pijoan, C. 1975. The effects of Mycoplasma hyorhinis and of Mycoplasma hyopneumoniae on pig kidney primary tissue culture. Br. Vet. J. 131:586-594.

Pijoan, C. and E. Boughton. 1974. Tube agglutination tests with Mycoplasma hyopneumoniae and M. hyorhinis. Br. Vet. J. 130:593-597.

Pollock, M. E. and S. V. Bonner. 1969. Comparison of undefined medium and its dialyzable fraction for growth of Mycoplasma. J. Bacteriol. 97:522-525.

Priestly, F. W. 1952. Observations of immunity to contagious bovine pleuropneumonia with special reference to the bactericidal action of blood. Br. Vet. J. 108:161-163.

Pullar, E. M. 1948. Infectious pneumonia of pigs. I. General description, differential diagnosis and epidemiology. Aust. Vet. J. 24:320-330.

Purcell, R. H., R. M. Chanock and D. Taylor-Robinson. 1969. Serology of the mycoplasmas of man. Pp. 221-264 in L. Hayflick, ed. The Mycoplasmatales and the L-phase of bacteria. North-Holland Publishing Company, Amsterdam.

Purcell, R. H., D. Taylor-Robinson, D. Wong and R. M. Chanock. 1966. Color test for the measurement of antibody to T-strain Mycoplasmas. J. Bacteriol. 92:6-12.

Purcell, R. H., D. Wong, R. M. Chanock, D. Taylor-Robinson, J. Canchola and J. Valdesuso. 1967. Significance of antibody to mycoplasma as measured by metabolic-inhibition techniques. Ann. N.Y. Acad. Sci. 143:664-675.

Razin, S. 1968. Mycoplasma taxonomy studies by electrophoresis of cell proteins. J. Bacteriol. 96:687-694.

Razin, S. and S. Rottem. 1967. Identification of mycoplasma and other microorganisms by polyacrylamide-gel electrophoresis of cell proteins. J. Bacteriol. 94:1807-1810.

Razin, S., B. Prescott and R. M. Chanock. 1970a. Immunogenicity of Mycoplasma pneumoniae glycolipids: a novel approach to the production of antisera to membrane lipids. Proc. Natl. Acad. Sci. U.S.A. 67:590-597.

Razin, S., J. Valdesuso, R. H. Purcell and R. M. Chanock. 1970b. Electrophoretic analysis of cell proteins of T-strain Mycoplasmas isolated from man. J. Bacteriol. 103: 702-706.

Razin, S., B. Prescott, W. D. James, G. Caldes, J. Valdesuso and R. M. Chanock. 1971. Production and properties of antisera to membrane glycolipids of Mycoplasma pneumoniae. Infect. Immun. 3:420-423.

Ressler, N. 1960. Two-dimensional electrophoresis of protein antigens with an antibody containing buffer. Clin. Chim. Acta 5:795-800.

Rhoades, K. R., M. Phillips and H. W. Yoder. 1974. Comparison of strains of Mycoplasma gallisepticum by polyacrylamide gel electrophoresis. Avian Diseases 81:91-96.

Roberts, D. H. and T. W. A. Little. 1970. Serological studies in pigs with Mycoplasma hyopneumoniae. J. Comp. Pathol. 80:211-220.

Rodwell, A. W. and E. S. Rodwell. 1978. Relationships between strains of Mycoplasma mycoides subspp. mycoides and capri studies by two-dimensional gel electrophoresis of cell proteins. J. Gen. Microbiol. 109:259-263.

Rose, D. L., J. G. Tully and R. G. Wittler. 1979. Taxonomy of some swine mycoplasmas Mycoplasma suipneumoniae, Goodwin et al. 1965, a later objective synonym of Mycoplasma hyopneumoniae, Maré and Switzer 1965, and the status of Mycoplasma flocculare, Meyling and Friis 1972. Int. J. Bacteriol. 29:83-91.

Rosendal, S. 1973. Analysis of the electrophoretic pattern of mycoplasma proteins for the identification of canine mycoplasma strains. Acta Pathol. Microbiol. Scand. Sect. B 81:273-281.

Ross, R. F. and J. A. Karmon. 1970. Heterogeneity among strains of Mycoplasma granularum and identification of Mycoplasma hyosynoviae, sp. n. J. Bacteriol. 103:707-713.

Ross, R. F., H. Grebe and H. Kirchhoff. 1978. Serological and electrophoretic characteristics of Mycoplasma hyosynoviae. Zentralbl. Vet. Med. B 25:444-451.

Rottem, S. and S. Razin. 1967. Electrophoretic patterns of membrane proteins of Mycoplasma. J. Bacteriol. 94: 359-364.

Rottem, S., M. Hasin and S. Razin. 1973. Binding of proteins to mycoplasma membranes. Biochimica et Biophysica Acta 298:876-886.

Shope, R. E. 1931. Swine influenza. J. Exp. Med. 56: 349-359.

Subcommittee on the Taxonomy of Mycoplasmatales. 1975. Minutes of the meetings. Sept. 18, 1974. Int. J. Syst. Bacteriol. 25:237-239.

Switzer, W. P. 1955. Studies on infectious atrophic rhinitis, IV. Characterization of a pleuropneumonia-like organism isolated from nasal cavities of swine. Am. J. Vet. Res. 16:540-544.

Switzer, W. P. and R. F. Ross. 1975. Mycoplasmal diseases. Pp. 741-764 in H. W. Dunne and A. D. Leman, eds. Diseases of Swine. 4th ed. Iowa State University Press, Ames, Ia.

Takayama, K., D. H. Maclennan, A. Tzagoloff and C. D. Stoner. 1966. Studies on the electron transfer system. LX VII. Polyacrylamide gel electrophoresis of the mitochondrial electron transfer complexes. Arch. Biochem. Biophys. 114: 223-230.

Taylor-Robinson, D. and D. M. Berry. 1969. The evaluation of metabolic inhibition technique for the study of Mycoplasma gallisepticum. J. Gen. Microbiol. 55:127-137.

Taylor-Robinson, D., N. L. Somerson, H. C. Turner and R. M. Chanock. 1963. Serological relationships among human mycoplasmas as shown by complement-fixation and gel diffusion. J. Bacteriol. 85:1261-1273.

Taylor-Robinson, D., R. H. Purcell, D. C. Wong and R. M. Chanock. 1966. A color test for the measurement of antibody to certain Mycoplasma species based upon the inhibition of acid production. J. Hyg. (Camb.) 64:91-104.

Texier, J., C. Mouches, J. M. Bove, D. Taylor-Robinson, C. Howard, N. Gourlay and J. Latrille. 1980. Protein analysis on polyacrylamide gels as applied to the characterization of human and animal Ureaplasmas and the detection of differences between virulent and non-pathogenic strains of M. pulmonis. P. 57 in Abstracts, Third Conference of the International Organization for Mycoplasmology, Custer, South Dakota, Sept. 3-9.

Thirkill, C. E. and G. E. Kenny. 1974. Serological comparison of five arginine-utilizing Mycoplasma species by two-dimensional immunoelectrophoresis. Infect. Immun. 10:624-632.

Thirkill, C. E. and G. E. Kenny. 1975. Antigenic analysis of three strains of Mycoplasma arginini by two-dimensional immunoelectrophoresis. J. Immun. 114:1107-1111.

Tiong, S. K. 1981. Isolation and identification of Mycoplasmas from pig lungs in Singapore. Vet. Rec. 108: 75-77.

Weeke, B. 1973a. General remarks on principles, equipment, reagents and procedures. Pp. 15-35 in N. H. Axelsen et al., eds. A manual of quantitative immunoelectrophoresis. Methods and applications. (Scand. J. Immunol. Vol. 2, supplement No. 1.) Universitetsforlaget, Oslo.

Weeke, B. 1973b. Crossed immunoelectrophoresis. Pp. 47-56 in N. H. Axelsen et al., eds. A manual of quantitative immunoelectrophoresis. Methods and applications. (Scand. J. Immunol. Vol. 2, supplement No. 1.) Universitetsforlaget, Oslo.

Wegman, W., H. U. Bertschinger and H. Keller. 1969. Die enzootische Pneumonie der Schweine. Eine elektronemikroskopische Untersuchung mit Erregernachweis im Gewebe. Zentralbl. Vet. Med. B 16:428-447.

Wesslén, T. and N. Lannek. 1954. The isolation and cultivation in tissue culture of a cytopathogenic agent from pigs with enzootic pneumonia (so-called virus pneumonia). Nord. Vet. 6:481-499.

Whittlestone, P. 1957. Some respiratory diseases of pigs. Vet. Rec. 69:1354-1366.

Whittlestone, P. 1958. Enzootic pneumonia of pigs and related conditions. Ph.D. dissertation. University of Cambridge, England.

Whittlestone, P. 1973. Enzootic pneumonia of pigs (EPP). Adv. Vet. Sci. Comp. Med. 17:1-55.

Whittlestone, P. 1979. Porcine Mycoplasmas. Pp. 133-176 in J. G. Tully and R. F. Whitcomb, eds. The Mycoplasmas. Vol. II. Academic Press, New York.

Williams, M. H. and D. Taylor-Robinson. 1967. Antigenicity of mycoplasma membranes. Nature, London 215: 973-974.

Wilson, A. B. 1976. Microflora of pneumonic lungs in a pig herd established by hysterectomy. Res. Vet. Sci. 20: 36-39.

Wreghitt, T. G., G. D. Windsor and M. Butler. 1974. Flat gel polyacrylamide electrophoresis of porcine mycoplasmas. Applied Microbiol. 28:530-533.

Wroblewski, H. and D. Ratanasavanh. 1976. Étude par immunoélectrophorèse bidimensionelle de la composition antigénique de la membrane de quelques souches de mycoplasmes. Can. J. Microbiol. 22:1048-1053.

Wroblewski, H., K. E. Johansson and R. Burlot. 1977. Crossed immunoelectrophoresis of membrane proteins from Acholeplasma laidlawii and Spiroplasma citri. Int. J. Syst. Bacteriol. 27:97-103.

Yaguzhinskaya, O. E. 1976. Detection of serum proteins in the electrophoretic patterns of total proteins of mycoplasma cells. J. Hyg. (Camb.) 77:189-198.

Yamamoto, K., K. Koshimizu and M. Ogata. 1971. Selective isolation of Mycoplasma suipneumoniae from pneumonic lesions in pigs. Natl. Inst. Anim. Hlth. Quart. 11:168-169.

Zola, H., W. Baxendale and L. J. Sayer. 1970. Polyacrylamide gel electrophoresis of lysate of mycoplasmas. Res. Vet. Sci. 11:397-399.

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